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15962

A Comparison of Effect of Caronamide and Benzoic Acid on Penicillin Plasma Concentrations.

WILLIAM P. BOGER* AND RICHARD M. BAKER.[†] (Introduced by L. E. Arnow.)

From the Philadelphia General Hospital.

The excretion of penicillin by the renal tubules can be inhibited by diodrast,[1,2,3] para-aminohippuric acid,[3,4,5] benzoic acid and sodium benzoate,[6,7,8] and caronamide.[9,10,11] Although both diodrast and para-aminohippuric acid have been used clinically to increase penicillin plasma concentrations, the necessity of administering intravenously large quantities of the drugs has limited their general usefulness. Benzoic acid, sodium benzoate and caronamide can be administered orally and may therefore be more widely used in conjunction with penicillin therapy. Since no comparison between the effects of benzoic acid and the new compound, caronamide, has appeared in the literature, such a comparison is here reported.

Plan of Study. Seven individuals who were afebrile and without evidence of cardiac, renal, or hepatic dysfunction, were selected for this study. Each patient was studied during 3 periods and served as his own control. Hospital routine was not modified and no effort was made to limit the intake of food or fluid.

In order that assayable plasma concentrations of penicillin might be anticipated during the entire 3 hour period over which dose response curves were obtained, intramuscular doses of 200,000 units were employed. This dose was dissolved in 2 cc of sterile saline and the sodium salt of penicillin G[‡] was used exclusively. The penicillin dose given immediately before the determination of peni-

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† Resident in Medicine, Philadelphia General Hospital.

"Staticin" caronamide (4'-carboxyphenyl-methanesulfonanilide) used in this investigation was supplied through the courtesy of Sharp and Dohme, Inc.

1 Rammelkamp, C. H., and Bradley, S. W., PROC. SOC. EXP. BIOL. AND MED., 1943, 53, 30.

2 Mokotoff, R., Brams, W., Katz, L. N., and Howell, K. M., Am. J. Med. Sci., 1946, 211, 395.

3 Avery, N. L., Jr., Mayer, O. B., and Nelson, R. C., Ann. Int. Med., 1946, 24, 900.

4 Beyer, K. H., Flippin, H., Verwey, W. F., and Woodward, R., J. A. M. A., 1944, 126, 1007.

5 Loewe, L., Rosenblatt, P., and Alture-Werber, E., Am. Heart J., 1946, 32, 327.

6 Spaulding, E. H., Bondi, A., Jr., and Early, E., Science, 1947, 105, 210.

7 Bronfenbrenner, J., and Favour, C. B., Science, 1945, 101, 673.

8 Bohls, S. W., Cook, E. B. M., and Potter, R. T., J. Ven. Dis. Inf., 1946, 27, 69.

9 Beyer, K. H., Science, 1947, 105, 94.

10 Crosson, J. W., Boger, W. P., Shaw, C. C., and Miller, A. K., J. A. M. A., 1947, 134, 1528.

11 Boger, W. P., Kay, C. F., Eisman, S. H., and Yeoman, E. E., Am. J. Med. Sci., in press.

‡ Commercial Solvents Corporation, Lot No. 46080801, expiration date October, 1949, potency 1572 units/mg.

cillin dose response curves was injected into the right deltoid region by one of the authors and massage of the injection site was avoided.

The pattern of patient study was as follows: *Control day.* An intramuscular injection of 200,000 units of penicillin at 6 and 9 A. M. Following the 9 o'clock injection into the right deltoid region, blood specimens for penicillin assay were obtained at intervals of 15 minutes, 30 minutes, 1 hour, 2 hours and 3 hours (this schedule of sampling is referred to as a dose response curve). *Benzoic acid treatment day.* Each patient was given benzoic acid every 3 hours for 24 hours before a dose response curve was determined. At the same time that the 6 and 9 A. M. doses of benzoic acid were given, an intramuscular injection of 200,000 units of penicillin was administered. Following the 9 o'clock penicillin and benzoic acid medication a dose response curve was obtained. Benzoic acid was administered in 0.5 g gelatin capsules; patients JJ, PR, JC and JA received 2 g every three hours. Patients SB, JG and MV received 3 g every 3 hours. *Caronamide treatment day.* This was similar to the benzoic acid treatment day except that caronamide in 0.5 g tablets was administered in place of benzoic acid.

It should be pointed out that, (a) in all instances at least a three day interval intervened between the benzoic acid and caronamide treatment days; (b) in the case of patients JJ, PR, JC and JA the administration of benzoic acid preceded that of caronamide, whereas patients SB, JG and MV received caronamide before benzoic acid; and (c) patients JJ and SB were incompletely studied so that a caronamide treatment day is lacking for JJ and a benzoic acid treatment day is lacking for SB.

Methods. Blood specimens for penicillin assay were drawn into sterile syringes containing 1 cc of 0.4% sodium citrate solution and were promptly transferred to graduated 15 cc centrifuge tubes. The specimens were centrifuged for 15 minutes at 1500 rpm and the total volume as well as the packed cell volume were recorded. The supernatant plasma was harvested and refrigerated in contact

with ice and remained so until penicillin assay on the same or subsequent day. Assays were done by the modified [12] serial dilution method of Rammelkamp using a Group A hemolytic streptococcus as the test organism. All penicillin values recorded in Fig. 1 are corrected for the dilution factor introduced by use of the citrate anticoagulant.

Discussion. It is apparent from the data shown in Fig. 1 that caronamide had a striking effect upon penicillin plasma concentrations when administered orally in doses of 2 or 3 g every 3 hours. Equally apparent is the lack of any significant effect of benzoic acid when given in doses of 2 or 3 g every 3 hours. Six patients showed a marked increase of penicillin plasma concentrations in response to caronamide therapy and 6 patients failed to show any favorable response to benzoic acid treatment. The individual responses to caronamide and to benzoic acid were quite uniform but the averaged data are most striking (Fig. 1). Whereas the benzoic acid dose response curve corresponds exactly to that obtained during the control period, the dose response curve modified by caronamide shows fold increases: at 15 minutes—1.67, 30 minutes—3.57, 1 hr.—3.83, 2 hr.—5.35 and 3 hr.—11.68 (average 5.22) times the control values. The enhancement effects noted in this study are in good agreement with those previously noted. [10, 11]

That benzoic acid had little effect on penicillin plasma concentrations if patients were permitted to have an unrestricted diet was noted by Bronfenbrenner and Favour.[7] These authors sharply restricted fluid and salt intake so that "the urine volume commonly fell to 400 to 600 cc in 24 hours," and gave 2.5 g of benzoic acid in capsules every 4 hours. On this regimen, enhancement effects were demonstrated and were ascribed to benzoic acid.

The patients studied in our series were given food and fluid ad libitum and doses of 16 and 24 g of benzoic acid per day. No elevation of penicillin plasma concentrations

12 Beyer, K. H., Peters, L., Woodward, Roland, and Verwey, W. F., *J. Pharm. and Exp. Therap.,* 1944, 82, 312.

COMPARISON OF THE EFFECTS OF BENZOIC ACID AND CARONAMIDE UPON
PENICILLIN DOSE RESPONSE CURVES
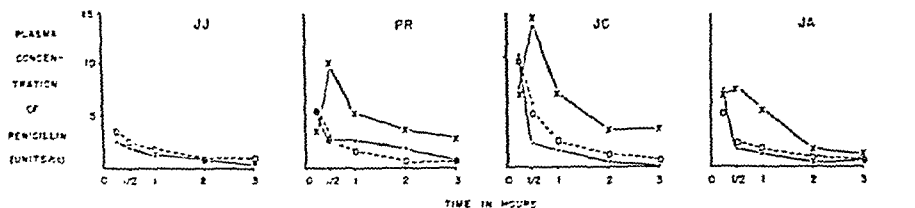
Effect of benzoic acid 2Gm/3hrs compared with effect of coronamide 2Gm/3hrs. (4 patients)
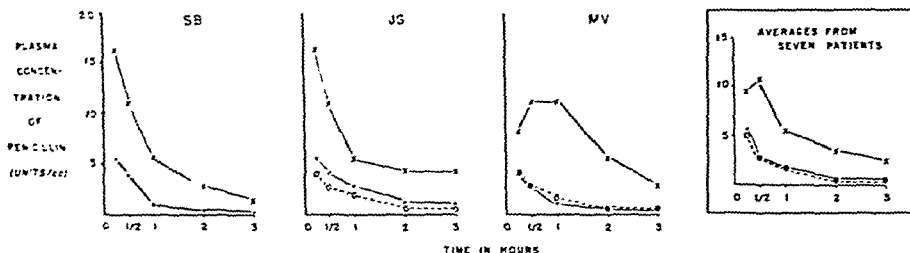
Effect of benzoic acid 3Gm/3hrs compared with effect of coronamide 3Gm/3hrs. (3 patients)

LEGEND

Penicillin, 200,000 u intramusc at zero time

——— penicillin alone

○----○ penicillin and benzoic acid

x——x penicillin and coronamide

Each patient was his own control. Several days intervened between the administration of benzoic acid and coronamide. Patients JJ, PR, JC, and JA were given benzoic acid before coronamide, patients SB, JG, and MV received coronamide before benzoic acid

FIG. 1.

was observed. It was suggested that the results reported by Bronfenbrenner and Favour are largely attributable to fluid and salt restriction. The desirability of lowering the daily urinary output to as little as 400 to 600 cc is open to question, especially if patients are suffering from febrile illness. If, however, there is no contraindication to fluid restriction, it can be anticipated that the enhancing effect of dehydration could be superimposed upon the effect of caronamide herein reported.

Spaulding, Bondi and Early[6] reported favorably upon the effect of sodium benzoate in elevating plasma concentrations following the oral administration of penicillin, unrestricted fluid and food intake, and a small dose of sodium benzoate. Bronfenbrenner and Favour indicated that, "on the normal diet, 2.5 g of benzoic acid equalled 6 g of sodium benzoate in raising serum penicillin level." Thus, on an unrestricted diet, Spaulding, Bondi and Early observed a positive effect of a small dose of sodium benzoate, whereas Bronfenbrenner and Favour using a larger dose of benzoic acid, which they regard as a more effective drug than sodium benzoate, observed only a slight effect.

The prolonged enhancing effect of sodium benzoate reported by Bohls, Cook and Potter[8] has been questioned by Spaulding, Bondi and Early on the basis of certain shortcomings of the penicillin assay methods used.

It is quite clear that the few data available on the effects of benzoic acid and sodium benzoate do not permit final evaluation of these two agents as adjuncts to penicillin therapy. If, as indicated by Bronfenbrenner and Favour, restriction of fluid is required to demonstrate an enhancing effect of benzoic acid, use of this agent would have certain disadvantages in the treatment of febrile diseases.

Hippuric acid is formed both in the liver and kidneys by the conjugation of benzoic acid and glycine. Although it is postulated that the hippuric acid thus formed acts in a manner similar to para-aminohippuric acid, it is extremely doubtful whether the amount of circulating hippuric acid following benzoic acid administration is sufficient to inhibit by "mass action" the tubular excretion of penicillin. It is conceivable that benzoic acid may be conjugated in the kidneys and give rise to a "local" high hippuric acid concentration, but this is not subject to proof at present.

In the light of the data here reported, it is our opinion that benzoic acid has no effect upon penicillin plasma concentrations when given in the amounts described.

Conclusions. Benzoic acid given in doses of 16 and 24 g per day to patients on unrestricted diets failed to increase penicillin plasma concentrations. Caronamide given to the same patients in the same doses showed a uniform and marked enhancing effect, averaging five times the control values.

15963

Further Studies of Effect of Sulphur Compounds on Production of Diabetes with Alloxan.

ARNOLD LAZAROW.   (Introduced by N. L. Hoerr.)

From the Department of Anatomy, Western Reserve University School of Medicine, Cleveland, Ohio.

In previous publications [1,2] it was reported that the intravenous injection of large doses of cysteine, glutathione, or thioglycolic acid completely protected rats against a diabetogenic dose of alloxan (40 mg/kg). This protection was observed only when the sulfhydryl compound was administered just prior to the alloxan, or within one minute after the alloxan injection. However if a large dose of cysteine was given 3 or more minutes following the alloxan, no significant protection was observed. It was further suggested that these extraneously administered compounds protected sulfhydryl enzymes from inactivation by alloxan. In this connection it is of interest to note that ascorbic acid, a compound which is reported to decrease the sulfhydryl groups in the body [3] potentiates the effect of alloxan.[4] The more effective production of diabetes in the starved rat [5] may also be related to variation in the glutathione and cysteine content of the body.

Since it was suggested [2] that alloxan may produce its diabetogenic effect by inactivating essential sulfhydryl groups of enzymes, it was desired to test other compounds with known sylfhydryl reactivating effects. The inhibiting effect of arsenic and cadmium on sulfhydryl enzymes has been reported by Peters [6] to be completely reversed by dimercaptopropanol, British Anti-Lewisite, (BAL).

[1] Lazarow, A., *Anat. Rec.*, 1945, 91, 24.

[2] Lazarow, A., PROC. SOC. EXP. BIOL. AND MED., 1946, 61, 441.

[3] Prunty, F. T. G., and Vass, C. C. N., *Biochem. J.*, 1943, 37, 506.

[4] Levey, S., and Suter, B., PROC. SOC. EXP. BIOL. AND MED., 1946, 63, 341.

[5] Kass, E. H., and Waisbrin, B. A., PROC. SOC. EXP. BIOL. AND MED., 1945, 60, 303.

[6] Peters, R. A., Stocken, L. A., and Thompson, R. H. S., *Nature, London,* 1945, 156. 616.

It was desirable to test this compound (BAL) for its antagonistic effect against alloxan diabetes. Also a number of non-sulfhydryl sulphur compounds was tested. Preliminary results have been reported.[7]

Methods. The protection experiments were carried out in the same manner as those previously reported.[2] Alloxan and the protective agent were administered intravenously, using separate tail veins for each injection. To facilitate the intravenous injection, vasodilatation was induced by placing the rats in a 56°C oven for one to several minutes. A 2% solution of alloxan monohydrate was used. The BAL was administered in doses of .16-1.00 mM/kg. The various dilutions of the BAL were prepared in .85% sodium chloride and were injected within 5 minutes of the time of preparation. In other experiments methionine was also used in doses of 2.9 mM/kg. A solution containing 0.25 mM of methionine per cc was injected in doses of 1.15 cc for each 100 g of body weight. This solution is near saturation. Thiourea was also used in doses of 7.5 mM/kg. One cc of a solution containing .75 mM per cc was used for each 100 g of body weight. The solution of thiourea had a pH of about 6.6, and was not neutralized, inasmuch as minute amounts of N/100 sodium hydroxide brings the pH to neutrality. Serial blood sugars were taken at 0, 3, 8, 24, 48, and 72 hours. The blood was obtained by cutting the tip of the tail, the rat being heated to facilitate bleeding; and the sugars were determined by the Folin-Malmros micro method.[8]

Results. The effects of administration of various sulphur compounds are given in Table I. The per cent of animals with diabetes are given for each group, and the average 48-hour blood sugars are listed for the diabetic and non-diabetic animals. An animal was considered diabetic if the 48-hour blood sugar was greater than 200 mg per 100 cc; or if the typical blood sugar response was observed following the injection of alloxan. All of 15 animals, injected with alloxan in doses of 40

[7] Lazarow, A., *Anat. Rec.*, 1947, 97, 37.
[8] Folin, O., and Malmros, H., *J. Biol. Chem.*, 1929, 83, 115.

TABLE I.
Effect of Various Sulphur Compounds on Production of Diabetes with Alloxan. Both alloxan and the protective agent were given intravenously.

Protective agent	No. of animals	Dose of alloxan mg/kg	Dose of protective agent mM/kg	Substance given first	Time between injections minutes	% diabetic	Avg 48-hr blood sugar Diabetic mg/100 cc	Avg 48-hr blood sugar Nondiabetic mg/100 cc
None	15	40	0			100	444	
BAL	4	40	.16	BAL	1-2	50	396	154
"	2	40	.36	"	1-2	0		150
"	2	40	.72	"	1-2	0		148
"	2	40	1.00	"	1-2	Dead		
"	4	0	.16					154
"	2	0	.36					150
"	1	0	.72					140
"	2	0	1.00			Dead		
"	1	40	.16	Alloxan	5	100	404	
"	4	40	.75	Alloxan	5	100	403	
Methionine	3	40	2.9	Methionine	1-2	100	412	
Thiourea	4	40	7.5	Thiourea	1-2	100	408	
"		40	7.5	"	1-2	Death 1-2 hr		
"		0	7.5	"		0		164

Hippuric acid is formed both in the liver and kidneys by the conjugation of benzoic acid and glycine. Although it is postulated that the hippuric acid thus formed acts in a manner similar to para-aminohippuric acid, it is extremely doubtful whether the amount of circulating hippuric acid following benzoic acid administration is sufficient to inhibit by "mass action" the tubular excretion of penicillin. It is conceivable that benzoic acid may be conjugated in the kidneys and give rise to a "local" high hippuric acid concentration, but this is not subject to proof at present.

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6 Peters, R. A., Stocken, L. A., and Thompson, R. H. S., *Nature, London*, 1945, **153**, 616.

Methionine was also studied because it is important as a methyl donator, and because it contains sulphur. Unfortunately, the solubility of methionine is limited to about .22 mM per cc at 25° C, and in order to inject a dose of 7.5 mM/kg, each rat (average weight of 350 g) would have to have been injected with about 10 cc of fluid intravenously. Consequently, the dose of methionine used in the present experiment was not greater than 2.9 mM/kg. At this dose, no effect was observed; whereas, at corresponding doses of cysteine and glutathione,[2] 100% protection was noted. Thiourea was also studied because of its sulphur content, inasmuch as its sulphur is not in the SH form. Although thiourea did not protect against alloxan dam-

age, some animals given thiourea and alloxan died within the first 1-2 hours. Thiourea alone was not toxic. The mechanism of death in these animals given both thiourea and alloxan has not been determined.

Conclusion. BAL, in doses of .36-.72 mM/kg completely protected rats against a diabetogenic dose of alloxan (40 mg/kg intravenously) when the protective agent was given immediately preceding the alloxan injection. However, no protection was observed when the disulfhydryl compound was given 5 minutes following the alloxan. Methionine, in doses of 2.9 mM/kg, and thiourea, in doses of 7.5 mM/kg, injected immediately preceding a diabetogenic dose of alloxan did not alter the course of alloxan diabetes.

15964

The Relation of Pteroylglutamic Acid to Metal-Porphyrin Enzymes.[*]

JOHN R. TOTTER, EDITH SIMS, AND PAUL L. DAY.

From the Department of Biochemistry, School of Medicine, University of Arkansas, Little Rock.

Data was reported recently [1, 2] and interpreted as suggesting that pteroylglutamic acid (PGA) and its conjugates are involved in the synthesis of the porphyrin portions of metalporphyrin enzymes. This communication offers additional data in support of that hypothesis.

For the experiments recorded here the organism *Streptococcus faecalis* (8043) was grown in the usual pteroylglutamic acid assay

medium,[3] but with the addition of either potassium cyanide, hydrogen peroxide, or caffeine. The H_2O_2 was diluted, filtered through a Berkefeld filter and added aseptically in the required quantity to sterile culture tubes containing appropriate volumes of medium. KCN was treated similarly, after neutralization with acetic acid. The caffeine was added before autoclaving. The final concentrations of the inhibitors in the culture tubes were as follows: H_2O_2, 0.012%; KCN, 0.05%; caffeine, 0.5% (Table I). After inoculation, turbidity was determined at various intervals with a Coleman spectrophotometer. Responses of the organism to stimulation with varying quantities of PGA as the limiting factor was determined with and without the added inhibitor. As a control procedure, similar experiments were conducted by varying the amount of Ca pantothenate and keep-

1 Steinkamp, R., Shukers, C. F., Totter, J. R., and Day, P. L., *Fed. Proc.*, 1947, 6, 295.

2 Totter, J. R., and Sims, E., *Fed. Proc.*, 1947, 6, 208.

3 Mitchell, H. K., and Snell, E. E., *Univ. of Texas Pub.*, 1941, No. 4137, 36.

mg/kg, developed diabetes. The average 48-hour blood sugar was 444 mg per 100 cc. When BAL was injected in doses of .16-1.0 mM/kg, immediately preceding a diabetogenic dose of alloxan, the response varied with the amount of BAL administered. Two of 4 rats were protected at a dose of .16 mM/kg of BAL. All animals were protected when the dose was .36-.72; whereas, at a dose of 1.0 mM/kg, the animals died within 2 to 6 hours. BAL alone produced no significant effect in doses of .16-.72 mM/kg, but one animal given a dose of 1.0 mM/kg also died. When the BAL was injected 5 minutes after the alloxan administration, no protection was observed, even with a dose of 0.75 mM/kg which is near the toxic dose for rats.

Methionine, in doses of 2.9 mM/kg (or thiourea in doses of 7.5 mM/kg) did not modify the diabetogenic dose of alloxan. Some animals given thiourea, in doses of 7.5 mM/kg, preceding a diabetogenic dose of alloxan, died within the first 1 to 2 hours, whereas all animals given thiourea without alloxan survived.

Discussion. Mole per mole, BAL, which is a disulfhydryl compound, is at least twice as effective in protecting rats against alloxan diabetes, as is cysteine or glutathione. Doses of .16 mM/kg of BAL protected half of the rats, and doses of .36 mM/kg protected all the rats. With cysteine doses of 1.0 mM/kg, it was previously reported [2] that 12% of the animals became diabetic; whereas, with a dose of .5 mM/kg, 66% of the rats became diabetic. However, allowing for the 2 SH groups in BAL, this compound is at least as effective per mole of SH as is cysteine. The small number of animals used in the BAL experiments do not permit more quantitative comparisons.

It will be noted that the diabetogenic effect of alloxan cannot be modified by BAL when the latter compound is given 5 minutes after the alloxan. This is in marked contrast to the effect of BAL on arsenic and cadmium inactivation of sulfhydryl compounds. Peters [6] has reported that BAL is effective when administered after the heavy metal poisoning has already taken place. Thus if the diabetogenic effect of alloxan is due to inactivation of the sulfhydryl groups of essential enzymes, these sulfhydryl groups cannot be reactivated by BAL. Very little information is available as to the mechanism of inactivation of sulfhydryl groups which takes place with alloxan. If the inactivation consists of merely oxidizing SH groups to S-S groups, one might expect BAL, cysteine, and glutathione to reactivate these groups. However, if the alloxan formed an addition product with the SH groups of enzymes, (as do quinones) this type of inactivation would not be expected to be reversed by glutathione or BAL. Further studies on the combination of alloxan with SH groups are needed.

A complete and irreversible inactivation of the sulfhydryl groups of the beta cells in the pancreas may be requisite to the production of diabetes with alloxan. Reversible sulfhydryl inactivators, such as arsenic, cadmium, etc., might not be expected to produce diabetes because of their reversibility.

The selective inactivation of essential sulfhydryl groups in the beta cells could be a result of a low glutathione content. Since glutathione can protect sulfhydryl enzymes from inactivation, the local concentration of glutathione might determine the sensitivity of cells to sulfhydryl inactivators. Unfortunately, the glutathione content of the beta cells in the islets has not yet been determined.

Inasmuch as alloxan produces its diabetogenic effect within the first 5 minutes of injection, the amount of alloxan reaching the beta cells within this time is a critical factor. Inasmuch as administered glutathione protects against alloxan diabetes, the glutathione, which normally is present in the beta cells, must also act as a protective factor. When this protective amount of glutathione disappears, the alloxan would then be free to act on the cell enzymes. The blood supply of a tissue will also determine the total amount of alloxan reaching the cells during the critical period following its injection, for in a given time, a highly vascular region, such as the islet tissue, may receive a much greater amount of alloxan than other less vascular tissues.

Methionine was also studied because it is important as a methyl donator, and because it contains sulphur. Unfortunately, the solubility of methionine is limited to about .22 mM per cc at 25° C, and in order to inject a dose of 7.5 mM/kg, each rat (average weight of 350 g) would have to have been injected with about 10 cc of fluid intravenously. Consequently, the dose of methionine used in the present experiment was not greater than 2.9 mM/kg. At this dose, no effect was observed; whereas, at corresponding doses of cysteine and glutathione,[2] 100% protection was noted. Thiourea was also studied because of its sulphur content, inasmuch as its sulphur is not in the SH form. Although thiourea did not protect against alloxan dam-age, some animals given thiourea and alloxan died within the first 1-2 hours. Thiourea alone was not toxic. The mechanism of death in these animals given both thiourea and alloxan has not been determined.

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3 Mitchell, H. K., and Snell, E. E., Univ. of Texas Pub., 1941, No. 4137, 36.

mg/kg, developed diabetes. The average 48-hour blood sugar was 444 mg per 100 cc. When BAL was injected in doses of .16-1.0 mM/kg, immediately preceding a diabetogenic dose of alloxan, the response varied with the amount of BAL administered. Two of 4 rats were protected at a dose of .16 mM/kg of BAL. All animals were protected when the dose was .36-.72; whereas, at a dose of 1.0 mM/kg, the animals died within 2 to 6 hours. BAL alone produced no significant effect in doses of .16-.72 mM/kg, but one animal given a dose of 1.0 mM/kg also died. When the BAL was injected 5 minutes after the alloxan administration, no protection was observed, even with a dose of 0.75 mM/kg which is near the toxic dose for rats.

Methionine, in doses of 2.9 mM/kg (or thiourea in doses of 7.5 mM/kg) did not modify the diabetogenic dose of alloxan. Some animals given thiourea, in doses of 7.5 mM/kg, preceding a diabetogenic dose of alloxan, died within the first 1 to 2 hours, whereas all animals given thiourea without alloxan survived.

Discussion. Mole per mole, BAL, which is a disulfhydryl compound, is at least twice as effective in protecting rats against alloxan diabetes, as is cysteine or glutathione. Doses of .16 mM/kg of BAL protected half of the rats, and doses of .36 mM/kg protected all the rats. With cysteine doses of 1.0 mM/kg, it was previously reported [2] that 12% of the animals became diabetic; whereas, with a dose of .5 mM/kg, 66% of the rats became diabetic. However, allowing for the 2 SH groups in BAL, this compound is at least as effective per mole of SH as is cysteine. The small number of animals used in the BAL experiments do not permit more quantitative comparisons.

It will be noted that the diabetogenic effect of alloxan cannot be modified by BAL when the latter compound is given 5 minutes after the alloxan. This is in marked contrast to the effect of BAL on arsenic and cadmium inactivation of sulfhydryl compounds. Peters [6] has reported that BAL is effective when administered after the heavy metal poisoning has already taken place. Thus if the dia-

betogenic effect of alloxan is due to inactivation of the sulfhydryl groups of essential enzymes, these sulfhydryl groups cannot be reactivated by BAL. Very little information is available as to the mechanism of inactivation of sulfhydryl groups which takes place with alloxan. If the inactivation consists of merely oxidizing SH groups to S-S groups, one might expect BAL, cysteine, and glutathione to reactivate these groups. However, if the alloxan formed an addition product with the SH groups of enzymes, (as do quinones) this type of inactivation would not be expected to be reversed by glutathione or BAL. Further studies on the combination of alloxan with SH groups are needed.

A complete and irreversible inactivation of the sulfhydryl groups of the beta cells in the pancreas may be requisite to the production of diabetes with alloxan. Reversible sulfhydryl inactivators, such as arsenic, cadmium, etc., might not be expected to produce diabetes because of their reversibility.

The selective inactivation of essential sulfhydryl groups in the beta cells could be a result of a low glutathione content. Since glutathione can protect sulfhydryl enzymes from inactivation, the local concentration of glutathione might determine the sensitivity of cells to sulfhydryl inactivators. Unfortunately, the glutathione content of the beta cells in the islets has not yet been determined.

Inasmuch as alloxan produces its diabetogenic effect within the first 5 minutes of injection, the amount of alloxan reaching the beta cells within this time is a critical factor. Inasmuch as administered glutathione protects against alloxan diabetes, the glutathione, which normally is present in the beta cells, must also act as a protective factor. When this protective amount of glutathione disappears, the alloxan would then be free to act on the cell enzymes. The blood supply of a tissue will also determine the total amount of alloxan reaching the cells during the critical period following its injection, for in a given time, a highly vascular region, such as the islet tissue, may receive a much greater amount of alloxan than other less vascular tissues.

Methionine was also studied because it is important as a methyl donator, and because it contains sulphur. Unfortunately, the solubility of methionine is limited to about .22 mM per cc at 25° C, and in order to inject a dose of 7.5 mM/kg, each rat (average weight of 350 g) would have to have been injected with about 10 cc of fluid intravenously. Consequently, the dose of methionine used in the present experiment was not greater than 2.9 mM/kg. At this dose, no effect was observed; whereas, at corresponding doses of cysteine and glutathione,[2] 100% protection was noted. Thiourea was also studied because of its sulphur content, inasmuch as its sulphur is not in the SH form. Although thiourea did not protect against alloxan damage, some animals given thiourea and alloxan died within the first 1-2 hours. Thiourea alone was not toxic. The mechanism of death in these animals given both thiourea and alloxan has not been determined.

Conclusion. BAL, in doses of .36-.72 mM/kg completely protected rats against a diabetogenic dose of alloxan (40 mg/kg intravenously) when the protective agent was given immediately preceding the alloxan injection. However, no protection was observed when the disulfhydryl compound was given 5 minutes following the alloxan. Methionine, in doses of 2.9 mM/kg, and thiourea, in doses of 7.5 mM/kg, injected immediately preceding a diabetogenic dose of alloxan did not alter the course of alloxan diabetes.

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The Relation of Pteroylglutamic Acid to Metal-Porphyrin Enzymes.*

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*From the Department of Biochemistry, School of Medicine, University of Arkansas,
Little Rock.*

Data was reported recently [1,2] and interpreted as suggesting that pteroylglutamic acid (PGA) and its conjugates are involved in the synthesis of the porphyrin portions of metal-porphyrin enzymes. This communication offers additional data in support of that hypothesis.

For the experiments recorded here the organism *Streptococcus faecalis* (8043) was grown in the usual pteroylglutamic acid assay medium,[3] but with the addition of either potassium cyanide, hydrogen peroxide, or caffeine. The H_2O_2 was diluted, filtered through a Berkefeld filter and added aseptically in the required quantity to sterile culture tubes containing appropriate volumes of medium. KCN was treated similarly, after neutralization with acetic acid. The caffeine was added before autoclaving. The final concentrations of the inhibitors in the culture tubes were as follows: H_2O_2, 0.012%; KCN, 0.05%; caffeine, 0.5% (Table I). After inoculation, turbidity was determined at various intervals with a Coleman spectrophotometer. Responses of the organism to stimulation with varying quantities of PGA as the limiting factor was determined with and without the added inhibitor. As a control procedure, similar experiments were conducted by varying the amount of Ca pantothenate and keep-

1 Steinkamp, R., Shukers, C. F., Totter, J. R., and Day, P. L., Fed. Proc., 1947, 6, 295.

2 Totter, J. R., and Sims, E., Fed. Proc., 1947, 6, 298.

3 Mitchell, H. K., and Snell, E. E., Univ. of Texas Pub., 1941, No. 4137, 36.

TABLE I.

Growth of *Streptococcus faecalis* in Presence of Inhibitors, with Varying Concentrations of Pteroylglutamic Acid (PGA) or Calcium Pantothenate (CaP).

PGA mγ/ml	CaP γ/ml	Incubation time* hr	Caffeine %	KCN %	H$_2$O$_2$ %	Optical density
0.2	0.1	24	0.5	0	0	0
0.5	,,	,,	,,	,,	,,	.04
1.0	,,	,,	,,	,,	,,	.11
5.0	,,	,,	,,	,,	,,	.19
10.0	,,	,,	,,	,,	,,	.25
100.0	,,	,,	,,	,,	,,	.55
10	0.01	48	0.5	0	0	.48
10	0.5	,,	,,	,,	,,	.35
10	1.0	,,	,,	,,	,,	.55
10	5.0	,,	,,	,,	,,	.48
10	0.2	24	0	0.05	0	.36
10	0.5	,,	,,	,,	,,	.46
10	1.0	,,	,,	,,	,,	.48
10	5.0	,,	,,	,,	,,	.43
0.2	0.1	32	0	0.05	0	.01
0.5	,,	,,	,,	,,	,,	.07
1.0	,,	,,	,,	,,	,,	.09
5.0	,,	,,	,,	,,	,,	.51
10.0	,,	,,	,,	,,	,,	.55
100.0	,,	,,	,,	,,	,,	.66
0.2	0.1	48	0	0	0.012	.02
0.5	,,	,,	,,	,,	,,	.00
1.0	,,	,,	,,	,,	,,	.06
5.0	,,	,,	,,	,,	,,	.15
10.0	,,	,,	,,	,,	,,	.17
100.0	,,	,,	,,	,,	,,	.67

* The time required for initiation of growth varies with inoculum size, concentration of inhibitor, and concentration of PGA. The incubation times given were chosen so that growth values were intermediate between no growth and complete growth. In the absence of inhibitor, complete growth (optical density = 0.77) was reached at all concentrations of PGA above 1.0 mγ/ml in 18 hours.

ing the PGA concentration constant.

Table I gives representative values for the growth of the organisms (optical density) at appropriate times. It may be seen that the inhibition induced by KCN, H$_2$O$_2$, or caffeine was partially reversed by the addition of large quantities of PGA. On the other hand the addition of Ca pantothenate above the normal requirement for this factor was without beneficial effect. It should be pointed out that in the presence of H$_2$O$_2$ low concentrations of PGA eventually permitted nearly complete growth, indicating that there was little or no destruction of the vitamin by the peroxide.

Probably the only biochemical property the three inhibitors have in common is their relation to catalase, peroxidase, cytochrome, cyto-chrome oxidase, and perhaps other metal-porphyrin systems; it therefore seems logical to conclude that PGA acts by stimulating the production of extra quantities of metal-porphyrin enzymes.

The molarity of KCN required to bring about complete inhibition is of the same order of magnitude as that required to reduce the oxidation potential of hemin to a minimum, as shown by Barron.[4]

Keilin has studied the effect of caffeine on hematins and concluded that the purine forms complexes with porphyrin.[5] According to Cheyney [6] the inhibitory action of caffeine

[4] Barron, E. S. G., *J. Biol. Chem.*, 1937, **121**, 285.

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on fertilized *Arbacia* egg respiration is mediated through the cytochrome-cytochrome oxidase system. The percentage of caffeine herein found to inhibit *S. faccalis* is very similar to that found by Cheyney to inhibit cell division in *Arbacia*. These considerations would appear to offer strong support to the hypothesis suggested in this and earlier communications.

Summary. *Streptococcus faccalis* was grown in the usual PGA-deficient medium containing graded amounts of PGA, to which either 0.05% potassium cyanide, 0.5% caffeine, or 0.012% hydrogen peroxide was added as an inhibitor. The inhibition induced by any of these three substances was partially reversed by large amounts of PGA. In control experiments where the Ca pantothenate content of the medium was varied but the PGA content kept constant, no such reversal of inhibition was found even with high levels of Ca pantothenate. These data lend additional support to the suggestion that PGA is involved in the synthesis of the porphyrin portions of metal-porphyrin enzymes.

15965

Habitat of *Endameba buccalis* in Lesions of Periodontoclasia.[*]

Charles C. Bass.

From the School of Medicine, Tulane University of Louisiana, New Orleans, La.

Many years ago Johns and I[1] showed that *Endameba buccalis* are most numerous at the very bottom of the pyorrhoea pocket. Later Kofoid[2] substantiated, and extended the application of this observation.

Studies of amebae in periodontoclasia have been based largely upon material taken from the lesions around and between teeth *in situ*. Although dental literature contains large numbers of illustrations of sections of teeth, including the periodontal tissues in all stages of periodontoclasia, the amebae present usually have been overlooked. Probably this has resulted from the fact that they are located within the bacterial film on the tooth and are not easily recognized in sections prepared in the usual way. Kofoid and Hinshaw[3] reported the distribution of the amebae found at different levels in relation to the calculus in sections of two incisors removed at biopsy.

More recently, employing an entirely different method, I have been able to ascertain, more accurately, the location and habitat of the parasite in these lesions. The method consists essentially of microscopic study of material removed, under the dissecting microscope, with delicate micro-instruments, from different locations on extracted teeth which have been stained to facilitate indentification of the structures and material present.

Elsewhere I[4] have described a previously unrecognized demonstrable line on extracted teeth which indicates the location of the outer border of the epithelial attachment. It is called the "zone of disintegrating epithelial-attachment cuticle" or zdeac. This line not only indicates the location of the outer border of the epithelial attachment, but it also accurately indicates the location, on the tooth, of the very bottom of the periodontoclasia lesion. With the zdeac as a guide, small particles of the soft bacterial film material can be picked from any selected areas and

1 Bass, C. C., and Johns, F. M., *J. A. M. A.*, 1915, 64, 553.

2 Kofoid, C. A., *J. Paras.*, 1929, 15, 151.

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locations at and near the bottom of the lesion.

Technic. The equipment and technical procedures previously described[4] are required. Safranin (0.5% in water) is satisfactory for staining tooth specimens for the present purpose. Immerse the formalin preserved specimen in the stain 3 to 5 minutes or longer. Wash well in water and examine under the dissecting microscope. The zdeac is not sharply shown on specimens stained with safranin but it can be recognized satisfactorily by one who has previously familiarized himself with this important landmark.

For removal of particles of material for examination under higher magnifications, a suitable micrurgic blade is required. This is made[4] from a very fine needle, the point of which is ground to a thin blade or chisel shape, not more than 0.25 mm wide.

With the specimen in the field of the dissecting microscope and a good light focussed upon it from above, small particles are picked from the thin edge of the soft stained material extending down to, and sometimes overlapping, the outer border of the zdeac. One or more such selected particles are transferred to a droplet of 50% glycerine on a slide and there teased apart, if desired, with the aid of 2 very fine pointed needles. A ¼ size coverglass is put on but not pressed down too hard. The mounted specimen is now ready for examination.

By proper adjustment of the light, different levels around the thin edge of particles and for some distance inward, can be focussed well enough to recognize the amaebae and the filaments and branches of the leptotrichia among which they are imbedded.†

The Periodontoclasia Lesion. The lesion of periodontoclasia consists of a pocket or space ("pyorrhoea pocket") at various locations

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† The amebae are imbedded in a mass of bacterial material. This must be stained lightly with a weak stain to permit focussing upon them through the mass.

These amebae have not been generally observed in paraffin or celloidin sections of teeth and parodontal tissues. Perhaps this explains, to some extent, why the habitat of this widely distributed parasite has not been generally recognized in such material heretofore.

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about the tooth, and may extend all way or only part way around it. There is much variation in the depth of lesions around different teeth, and that of the lesions at different locations around the same tooth.

On one side of the lesion there is an inflamed suppurating surface of epithelial tissue extending from the gingival margin to the bottom of the lesion. On the opposite side is the tooth which is covered with more or less hard calculus. Attached to the calculus and to the tooth near it, not yet covered with calculus, there is a pad of soft bacterial material consisting of a compact mass, of variable thickness, of stems and filaments extending outward towards the space and downward towards the very bottom of the lesion. This latter has been noted recently by Box,[5] not, however, with any reference to amebae present.

The outer part of the pad attached to the tooth consists largely of radiating filaments which protrude at the surface as a thick-set carpet-like pile of growing, branching and fruiting stems. It is possible that the compact portion of the baterial pad attached to the tooth may consist of several different kinds of organisms of this type. However, the fruiting heads on the surface and the stems that can be focussed deeper in conform, in most instances, to *Leptothrix falciformis*. This organism was first described in material from around teeth by Buest[6] and given the name *L. falciformis*, because of the scythe- or blade-shaped conidia produced on the fruiting branches. These conidia or spore bearing curved to straight rods of varying size radiate, at an angle, from the central stalk, which is also surrounded by a large amount of jelly-like material in which the falciforms are imbedded.

Association of the Amebae with Leptotrichia and their Distribution. Intimate association of *E. buccalis* with filamentous bacterial material has been observed.[3,7,8] Good-

5 Box, H. K., *J. Canadian D. A.*, 1947, **13**, 3.

6 Buest, Theo. Von, *Dent. Cos.*, 1908, **50**, 594.

7 Barrett, M. T., *Dent. Cos.*, 1914, **56**, 948.

8 Goodrich, H. P., and Mosely, M., *J. Roy. Mic. Soc.*, 1916, Dec., 513.

Fig. 1. *E. buccalis* mounted, for comparison, by the side of an isolated fruiting stem of *L. falciformis.* × 1200.

Fig. 2. Outer edge of leptothrix bed; fruiting head covered with spirochetes; arrows point to ameba at edge and others, not sharply focussed, deeper in. × 810.

Fig. 3. Leptothrix bed; arrows point to amebae, partially focussed. × 810.

Fig. 4. Deep leptothrix bed; arrows point to imbedded amebae. × 810.

rich and Mosely[8] found that these amebae *(Entameba gingivalis)* are in greatest numbers on the under side of the tartar ridge. They claim that the parasites do not burrow into the tissues of the gum but often between the terminal branches of the leptotrichia which are found in abundance in the pyorrhoea lesion.

The space between the leptotrichial bed on the one side and the inflamed epithelial wall on the other, contains inflammatory tissue exudate, large numbers of bacteria of many varieties, spirochetes and usually some amebae which have come out from their bed to their feeding ground where there are an abundance of pus cells upon which they feed. After feeding the parasite usually withdraws into the leptothrix bed for safety and protection. Individuals that venture too far away from the bed into the open space are unable to return and are swept out with the pus, especially when it is squeezed out by pressure upon the tooth in chewing, biting, etc.

The parasites are found scattered among the branches and fruiting heads (Fig. 1) of the leptothrix bed. The individual not only burrows between the different elements— stems, filaments, falciforms—making up the outer surface, but apparently they also burrow about in the abundant jelly-like material imbedding these elements (Fig. 2, 3, 4). In studying several hundred such specimens as suggested here, I have often observed several amebae clustered about a leptothrix stalk and especially in the fork where a large stalk apparently divides into 2 smaller ones. For the most part, however, they are found separate and not in direct contact with each other.

Summary. A simple method is given, of collecting material from the area on extracted teeth, which is inhabited by *E. buccalis.*

The habitat of *E. buccalis* is the outer part of the filamentous bacterial film on the tooth, within the periodontoclasia lesion. There they are protected and live, grow and multiply among the strands and fruiting heads of leptotrichia, principally *L. falciformis.*

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Creeping Eruption Caused by the Larvae of the Cattle Hookworm
Bunostomum phlebotomum.

ROY L. MAYHEW.

From the College of Agriculture, Louisiana State University.

Several years ago while making a series of inoculations with third stage larvae of the hookworm, *Bunostomum phlebotomum*, and the nodular worm, *Oesophagostomum radiatum*, by placing the larvae on the skin of the calves we frequently noted the appearance of small inflamed spots between our fingers.[1] Some of these spots increased after a few hours to about ¼ inch in diameter, followed after 2 or 3 days by the appearance of a narrow, linear, tortuous eruption which became extended at intervals during the next few days. Following the course of these eruption areas a slightly raised, vesicular line generally developed within a few hours which

might be interrupted at points. The entire area became more or less swollen and intensely itchy particularly in the mornings. Within about 2 weeks the surface of the skin was dry, scaly, and the irritation gone. Recently another series of inoculations were undertaken using pure cultures of hookworm larvae[2] and we again noted the appearance of these spots always coincidently with the skin inoculations on the calves.

The following may be taken as a typical

1 Mayhew, R. L., *Cornell Vet.*, 1939, **29**, 367.

2 Mayhew, R. L., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 360.

description of one of these lesions. On October 3, 1946 several lesions developed following the application of larvae to the skin of a calf, one of these being on the interdigital surface of the ring finger on the side adjacent to the middle finger and at the distal joint. This lesion developed to the diameter of ½ inch in 2 or 3 hours, and remained in this condition until the night of October 7, when a narrow, elevated, linear extension about ¼ inch long appeared directed toward the palmar side of the finger. On the night of October 8 a right angle extension about ¼ inch long developed toward the tip of the finger. Between 6 and 9 P. M. on October 9 the eruption area was extended toward the palmar side of the finger about ¾ inch and back almost to the starting point in the form of an irregular oval. On the night of October 10 the eruption area was again extended about ¼ inch toward the tip of the finger. The entire area became swollen and was very itchy especially in the mornings. A slightly raised line, interrupted in places, and becoming vesicular in places after a few hours, developed along the eruption area. This vesicular line possibly represents the path of migration of the larvae. No additional migration was observed and by October 12 the swelling and irritation was beginning to disappear. In about another week the area no longer showed irritation, the swelling was gone, and the outer layer of skin was scaling off along the course of migration.

In our experience there was no evidence that the larvae penetrated by way of the hair follicles since no eruption areas appeared on the back of the fingers or hand where hair follicles are present but always on the relatively thin skin areas between the fingers. Two instances were noted in which larvae penetrated on the palm side of the fingers. One of these was on the palm side of the thumb near the tip and the other between the first and second joints of the left index finger. In each instance the skin had been punctured and slightly torn a day or two before and there was migration over ½ to ¾ inch area.

In a few instances a tingling or prickling sensation was noted. One of these we were able to observe in particular. A drop of the

Fig. 1.
Photograph showing eruption area in which 4 and possibly 5 larvae had penetrated. The linear eruptions of 2 of the larvae extended around on to the palmar side of the finger.

larval suspension was observed to run along between the index and middle finger of the left hand as they were held together, and within a few seconds a distinct prickling sensation was noted. That the calf has similar sensations is indicated by the fact that they have been observed many times to twitch the skin of the area to which the larvae are being applied during the next few minutes after contact with the suspension, and also to switch the tail toward the side on which the larvae had been applied.

The lesions and symptoms are in the main identical with those reported by Kirby-Smith, Dove, and White,[3] and White and Dove,[4] as developing from larvae of the dog hookworm, *Ancylostoma brazilense,* in man, and known as creeping eruption. The principal difference seems to be that the duration of the eruptions and irritation is not so prolonged in the case of the cattle hookworm. In our experience with the cattle hookworm the dura-

3 Kirby-Smith, J. L., Dove, W. E., and White, G. F., *Arch. Derm. Siph.,* 1926, 13, 137.
4 White, G. F., and Dove, W. E., *J. Am. Med. Assn.,* 1928, 90, 1701.

tion is one to three weeks while that of the dog hookworm is from several weeks to months. We also have the impression that the larvae of the cattle hookworm do not penetrate is as large numbers as those of the dog hookworm. In the instances coming under our observation there was no secondary infection while in the case of the dog hook-

worm larvae this may develop as a result of the prolonged scratching. There is much similarity to the schistosome dermatitis which has been so extensively studied in recent years by Cort,[5] and his associates. There is, however, no migration in schistosome dermatitis.

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[5] Cort, W. W., *Am. J. Hyg.*, 1936, **23**, 349.

15967·

Histochemistry XIX. Localization of Alkaline Phosphatase in Normal and Pathological Human Skin.*

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The present study was undertaken in the hope of establishing the histo- and cytologic distribution of alkaline and acid phosphatase and lipase in normal and pathologic human skin. Our results were essentially negative for the acid phosphatase and lipase, so that this report deals with alkaline phosphatase alone. The methods of Gomori[1] for the demonstration of the enzymes were employed throughout. All tissue sections were placed in 0.1 M citrate buffer, pH 4.5-5.0, for 30 minutes before the enzyme staining reaction was applied in order to remove preformed phosphates and other substances which might give a positive reaction. Inasmuch as control experiments on these sections demonstrated no staining whatsoever in the absence of substrate, any staining obtained could be ascribed to enzyme action alone. An incubation period of 12-24 hours in the substrate medium was used. In this study 32 specimens of normal skin, collected from 27 individuals, were selected from the scalp, face, breast, axilla, chest, palm, back, abdomen, penis, scrotum and hip. This selection was made because of the differences in histologic

structure of skin from those sources. The 33 pathological specimens were biopsied from 8 cases of eczema, 2 cases of lupus erythematosus, 2 cases of psoriasis, 1 case of papular urticaria, 2 cases of pyogenic granuloma, 15 cases of healing wounds and scars, and 3 cases of acne vulgaris.

In normal skin, alkaline phosphatase activity was demonstrated in all specimens. There was slight staining in the stratum granulosum in agreement with the findings of Bourne and MacKinnon[2] who investigated guinea pig skin. The endothelial walls of the capillaries showed the most uniform and clear cut staining effects as previously observed by Gomori[3] and others. The reaction was most pronounced in the nuclei of the endothelial cells and the nucleoli showed a stronger reaction than the other parts of the nuclei. The hairs and hair follicles, especially the papillae, gave an intense reaction in much the same manner as found for guinea pig skin by Johnson et al.,[4] and Bourne and MacKinnon.[2] However, in the present investigation, a

[1] Gomori, G., *Am. J. Clin. Path.*, 1946, **16**, 347.

[2] Bourne, G., and MacKinnon, M., *Quart. J. Exp. Physiol.*, 1943, **32**, 1.

[3] Gomori, G., *J. Cell. and Comp. Physiol.*, 1941, **17**, 71.

[4] Johnson, P. L., and Bevelander, G., *Anat. Rec.*, 1946, **95**, 193.

Fig. 1.
Section of axillary skin × 75. Showing slight enzyme activity in stratum granulosum and intense reaction in follicular papilla, capillaries and apocrine glands. (1) Follicular papilla, (2) apocrine glands, (3) sebaceous glands and surrounding vascular bed, (4) capillaries, (5) stratum granulosum.

greater intensity of staining was observed at the base of the follicular papillae which is the site of the nutrient capillary (Fig. 1). This was not previously described in the work on guinea pig skin. The only evidence of alkaline phosphatase activity we were able to demonstrate associated with the sebaceous glands was in the rich capillary bed surrounding them (Fig. 1). This is in contrast to the reports dealing with the work on guinea pigs[2] in which definite staining of sebaceous glands was reported.

Although previous reports have not mentioned alkaline phosphatase in the sweat glands, these glands showed marked activity with some variation in intensity in the loops. The activity in the apocrine glands was more clearly and uniformly demonstrable than in the eccrine glands. In both, the stain was

fairly diffuse but it was most prominent at the periphery of the cell and in the nucleus (Fig. 2). The heavier staining of the nucleolus as compared to the rest of the nucleus was most clearly apparent in the apocrine cell (Fig. 3).

Among the dermatologic states found to show no abnormal alkaline phosphatase activity are lupus erythematosus, psoriasis, and papular urticaria. Three specimens from different patients with acne vulgaris showed some diffuse deeper staining involving the lymphocytic infiltrate. The greatest change was seen in scar tissue or tissue showing evidence of new fibroblastic proliferation. In two biopsies obtained a few days after closure of a burn wound the nuclei of the fibroblasts gave a stronger reaction than the cellular cytoplasm. Three older scar tissues (2-8

Fig. 2.
Section of eccrine glands × 300. Nuclei, nucleoli and cell walls show positive reactions. The lighter staining sweat duct with its multiple layered cell wall can be seen.

months after healing of an operative wound) showed very definite, though slightly diminished, alkaline phosphatase activity in the fibroblasts (Fig. 4). *Still older scars of the same type contained a few scattered groups of fibroblasts giving the positive reaction.* These results are in accord with the observations of Fell and Danielli[5] who described the distribution of alkaline phosphatase in the fibroblasts and new collagen fibers of healing experimental wounds in rats. In the same year, Gomori reported phosphatase positive fibroblasts in connective tissue subjacent to newly formed epithelium in fascial transplants of bladder epithelium.[6] In a pyogenic granuloma the new fibroblasts reacted in the same fashion as those in scar tissue. The infiltrate, consisting of polymorphonuclear leucocytes and lymphocytes also gave a positive phosphatase reaction.

A study of the skin in 3 cases of eczema and in 3 cases showing positive allergic skin tests was made. *Sections from the sites of positive allergic reactions showed no abnormal change nor did those from relatively acute eczemas.* In 3 cases of long standing eczema with infiltration and thickening of the skin the picture was quite different (Fig. 5). Here the phosphatase reaction of the perivascular infiltrate, made up of lymphocytes, monocytes and some plasma cells, was distinctly positive. There was also some diffuse staining of the connective tissue including some of the fibroblasts in the deeper cutis.

Summary. In normal skin, alkaline phosphatase activity was observed in the stratum granulosum, endothelial lining of the capillaries, in hairs, hair follicles, and sweat glands. In the hair follicles, the greatest activity was

5 Fell, H. B., and Danielli, J. F., Brit. J. Exp. Path., 1943, **24**, 196.

6 Gomori, G., Am. J. Path., 1943, **2**, 197.

Fig. 3.
Section of apocrine glands × 300. The deeper
staining of the nucleoli is apparent.
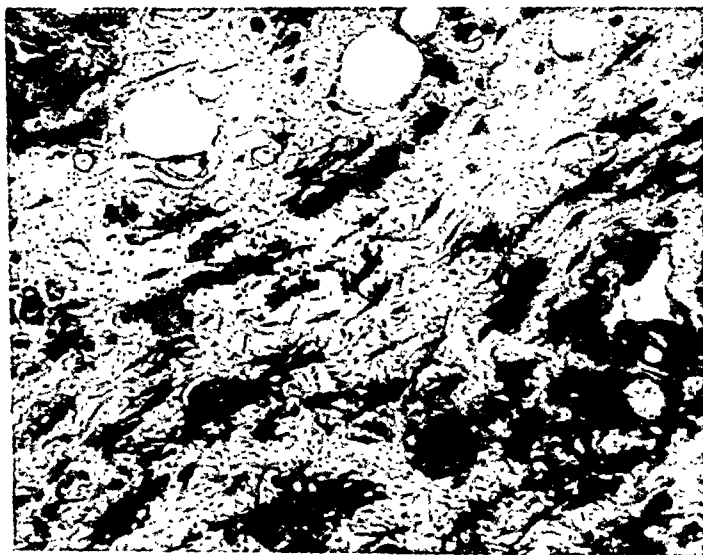
Fig. 4.
Section through a scar (8 mos.) × 150. Enzyme activity pronounced in fibroblasts
and connective tissue fibers.

Fig. 5.
Section of chronic eczema. In the deeper cutis,
proliferative activity is manifested by the pres-
ence of alkaline phosphatase in the new fibroblasts
and in the connective tissue fibers. Staining of
some of the infiltrate is also shown.

in the papillae. In the endothelial cells and cells of the sweat glands, the enzyme appears to be concentrated at the cell walls and in the nuclei, the nucleoli showing even greater activity than other parts of the nuclei.

Pathologic tissue from cases of lupus erythematosus, psoriasis and papular urticaria showed no significant changes in the enzyme activity.

Alkaline phosphatase activity was found in proliferating fibroblasts in scar tissue, especially in the newly formed scar, and also in the fibroblasts and infiltrate of pyogenic granuloma. The nuclei gave a more intense reaction than the cytoplasm of the cells.

In chronic eczema of long standing, the cellular perivascular infiltrate of lymphocytes, monocytes and plasma cells, as well as the fibroblasts, showed significant alkaline phosphatase activity.

In acne vulgaris, the infiltrate exhibited the enzyme activity.

15968

Cobalt Polycythemia. II. Relative Effects of Oral and Subcutaneous Administration of Cobaltous Chloride.

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(Introduced by Arthur A. Hellbaum.)
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In spite of the fact that the efficacy of cobalt salts in stimulating erythropoiesis is well established, cobalt therapy is little used in the treatment of anemia.[1] This is probably due to the fact that many experimenters have given cobalt salts to animals by subcutaneous injection, whereas oral administration should be preferred for human use. Also, there are few data concerning relative toxicity. No data have been recorded concerning the relative effects of cobalt salts on erythrocyte and hemoglobin production when administered orally, in contrast to subcutaneous injection. It has therefore been impossible to evaluate the effects of subcutaneous injection in terms of effective oral dosage. Approximately 4 times as much cobalt is excreted in the urine within the first 72 hours following subcutaneous administration as when the same amount is given orally.[2,3] This difference is apparently related to the difference in rates of absorption.

In order to determine the effects of cobalt on erythrocyte and hemoglobin production under conditions of oral versus parenteral administration, and to establish further the optimal and minimal toxic dosage of this substance, the following experiment was devised. Of 30 albino rats, Sprague-Dawley strain, weighing approximately 250 g each, 24 received cobaltous chloride;* 6, untreated, served as controls. The group of animals to be treated was subdivided into 6 groups of 4 each. Group 1 received 2.5 mg/kg $CoCl_2 \cdot 6H_2O$ daily by mouth. Group 2 received 2.5 mg/kg daily by subcutaneous injection. Groups 3 and 4 received 10 mg/kg daily, orally and by subcutaneous injection, respectively. Groups 5 and 6 received 40 mg/kg daily, orally and by subcutaneous injection, respectively.

Each dose of cobalt salt was injected subcutaneously in a solution of 0.2ml of distilled water. For oral administration, a solution of the cobalt salt was mixed with equal parts of wheat flour and powdered sugar to form a stiff paste and packed in No. 5 gelatin capsules. Just before administration the capsules were moistened and then inserted far back in the animal's pharynx. This method allowed a careful control of the oral dosage both as to amount and time of administration.

The experiment was continued for 8 weeks. Blood counts and hemoglobin determinations were made at the beginning of the experiment and at 2-week intervals. These data are summarized in Table I. It is apparent that 2.5 mg $CoCl_2 \cdot 6H_2O$/kg/day, given subcutaneously, effected an increase of 30% in the number of erythrocytes per cu mm of blood in 6 weeks. It required approximately 40 mg/kg/day of cobaltous chloride by mouth, or 16 times as much to produce a comparable increase in erythrocytes. Animals which received 10 mg/kg of cobaltous chloride daily by the subcutaneous route presented a more prompt erythrocyte and hemoglobin response. A daily dose of 2.5 mg/kg produced an essentially

1 Stanley, A. J., Hopps, H. C., and Hellbaum, A. A., PROC. SOC. EXP. BIOL. AND MED., 1946, 61, 130.

2 Greenberg. D. M., Copp, D. H., and Cuthbertson, E. M., *J. Biol. Chem.*, 1943, 147, 749.

3 Sheline, G. E., Chaikoff, I. L., and Montgomery, M. L., *Am. J. Physiol.*, 1946, 145, 285.

* Preparations of $CoCl_2 \cdot 6H_2O$ labeled "Reagent" vary considerably in their content of impurities. At least one such preparation contained sufficient lead to produce lead intoxication if given in amounts described here. We have restricted our use to the preparation marketed by Eimer and Amend and labeled "Cobalt Chloride C. P. $CoCl_2 \cdot 6H_2O$ Mol. wt. 237.95 Cat. no. C-371."

TABLE I.

Relative Effects of Cobaltous Chloride ($CoCl_2 \cdot 6H_2O$) on Production of Erythrocytes and Hemoglobin in Rats when Administered Orally and by Subcutaneous Injection.

No. animals	Daily dose mg/kg Oral	Subcut.	Erythrocytes in millions/cu mm. Time in weeks 0	2	4	6	8	Hemoglobin in g per 100 ml 0	2	4	6	8
4	2.5		8.84	9.07	8.90	9.56	8.64	16.2	16.2	17.0	17.0	17.2
4		2.5	8.65	9.44	10.33	11.38	11.65	16.1	16.4	19.4	21.8	22.5
4	10.0		9.03	9.76	10.28	10.58	10.40	16.2	16.6	18.2	20.0	20.0
4		10.0	9.36	10.07	12.59	11.98	11.73	16.4	18.2	22.2	22.9	22.4
4	40.0		8.83	9.25	10.05	11.17	11.42	15.9	17.0	18.6	20.8	21.7
4*		40.0	8.84	10.01	—	—	—	15.8	17.8	—	—	—
6	Controls		8.72	9.01	9.51	8.82	9.06	16.0	16.5	16.7	16.8	16.2

* None of these 4 animals survived beyond 8 days due to the toxicity of cobalt in such massive doses. Erythrocyte and hemoglobin determinations were made at the end of 6 days rather than at 2 weeks. The animals receiving the same dose orally survived.

similar effect, but required an additional 2 weeks.

Rats which received 2.5 mg/kg of cobaltous chloride subcutaneously or 40 mg/kg by mouth did not manifest significant toxic effects. Their body weight remained essentially constant, as did that of the controls. Rats which received 10 mg/kg of cobaltous chloride subcutaneously lost an average of 24% of their body weight by the end of 6 weeks. Those animals which received 40 mg/kg daily, subcutaneously, survived 5, 7, 8, and 8 days, respectively.

Summary. The relative effect of parenteral versus oral administration of cobaltous chloride on erythrocyte and hemoglobin formation in rats has been determined. Daily subcutaneous injection of 2.5 mg/kg of body weight, over a period of 6 weeks, resulted in an increase of more than 30% in the number of erythrocytes per cu mm and in grams of hemoglobin per 100 ml of blood. To produce a similar effect from oral administration of cobaltous chloride, within the same period of time, 40 mg/kg of body weight was required. These dosages are without significant toxic effects. Rats which received 10 mg cobaltous chloride/kg daily, subcutaneously, averaged 24% weight loss by the end of 6 weeks. Rats which received 40 mg/kg body weight, subcutaneously, did not survive beyond 8 days.

15969

Inhibition of the Hirst Haemagglutination Reaction by Pneumococcal Extract, Normal Serums, and Blood Cell Esterases.*

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During the course of other studies it was observed that culture filtrates of Type I and Type V pneumococci would inhibit the agglutination of chicken red cells by influenza virus. Stone[1,2] found that lecithinase would

[1] Stone, J. D., *Australian J. Exp. Biol. and Med. Sci.*, 1946, 24, 191.

[2] Stone, J. D., *Australian J. Exp. Biol. and Med. Sci.*, 1946, 24, 197.

TABLE I.

Substrate	Cu mm CO_2 evolved in 30 minutes							
	Cholinesterase from human A cells	Cholinesterase from human AB cells	Type I pneumo- coccus autolysate	Type V pneumococcus Autol- ysate	Filtrate	Normal rabbit serum	Normal G.P. serum	Normal human serum
Tributyrin .03 M	0	0	38	43	14	200	256	184
Acetyl choline .003 M	40	34	—	—	—	—	—	—

inhibit the hemagglutinating action of vaccinia and ectromelia viruses but did not affect the agglutination of red cells by influenza virus.

The discovery[6] of an esterase in pneumococcus cultures, subsequently identified as cholinesterase[7] suggested to us that the latter enzyme might bear a causal relationship to the inhibition of influenzal hemagglutination. While some of our results do not fit smoothly into such an explanation, they are not altogether contradictory and the data obtained seem of sufficient importance to warrant publication.

Materials and Methods. Pneumococcal esterase was prepared from Type I and Type V cultures grown in a beef heart infusion hormone broth. In one method of preparation, after 18-24 hours incubation the cultures were adjusted to pH 7 with N/1 NaOH, were refrigerated overnight (for increased autolysis) and then centrifuged. The supernatant was Seitz-filtered. A second preparation was obtained by repeated freezing and thawing (6 cycles) of saline suspensions of once-washed 18-hour cultures. In the following discussion the first preparation is designated as pneumococcal filtrate, the second as pneu-

mococcal autolysate.

For comparative purposes a partially purified specific cholinesterase was prepared from human red cells by the method of Mendel and Rudney.[8]

The inhibitory substances were titrated by a modification of the Hirst agglutinin-inhibition technic in which successive 2-fold dilutions of the inhibiting substance were substituted for serum dilutions. In all such tests 10 agglutinating units of PR8 influenza virus were employed per tube and a 0.75% chicken red-cell suspension was used instead of the heavier suspension recommended by Hirst. Naked-eye readings are facilitated by the use of the lighter cell suspensions. Inhibition titers are 2- to 4-fold higher than when 1.5% suspensions are used.

Experimental. Table I records the esterase activity of the above preparations and also of normal rabbit, guinea pig, and human sera. Schachter's modification[9] of the Ammon-Warburg technic was used with tributyrin as the substrate. Since the esterase of blood cells is a specific cholinesterase, acetyl choline was used in determining its activity.

In Table II are recorded the results of agglutinin-inhibition tests using normal human and animal sera as inhibiting agents as well as the esterases prepared from pneumococci and from human red cells.

To determine whether the effect of the pneumococcal inhibitory substance was due to action upon the virus, an unconcentrated allantoic fluid having a CCA titer of 1/1280 was mixed in equal proportions with Type

3 Friedewald, W., Miller, E. W., and Whatley, L. R., *Soc. Am. Bacteriologists, Abst. Proc.*, 47th meeting, 1947, 62.

4 Liebmann, A. J., Perlstein, D., and Snyder, G. A., *Soc. Am. Bacteriologists, Abst. Proc.*, 47th meeting, 1947, 63.

5 Woolley, D. W., and Green, R. H., *Soc. Am. Bacteriologists, Abst. Proc.*, 47th meeting, 1947, 63.

6 Avery, O. T., and Cullen, G. E., *J. Exp. Med.*, 1919, 29, 215; 1920, 32, 547, 571, 583; 1923, 38, 199.

7 Schaller, K. Z., *Physiol. Chem.*, 1942, 276, 271.

8 Mendel, B., and Rudney, H., *Biochem J.*, 1943, 37, 59.

9 Schachter, R. J., *Am. J. Physiol.*, 1945, 143, 552.

TABLE II.

Source of inhibiting material	Type I pneumococcus filtrate	Type I pneumococcus autolysate	Type V pneumococcus filtrate	Type V pneumococcus autolysate	Cholinesterase from human A cells	Cholinesterase from human AB cells	Normal G.P. serum	Normal rabbit serum	Normal human serum (Group O)	Normal human serum (Group AB)
Agglutinin-inhibition titer	1/16	1/128	1/256	1/512	1/32	1/64	1/512	1/2048	1/4096	1/512

V pneumococcal filtrate diluted 1/5 and the mixture was incubated at room temperature for 1⅛ hours. The virus thus treated was then diluted 1/128 with physiological saline to make a final virus concentration of 10 agglutinating units per cc and a final dilution of the filtrate of 1/640 (beyond the concentration at which it could inhibit agglutination). When a red cell suspension was added typical agglutination occurred. Virus similarly treated with red-cell cholinesterase or with guinea pig serum was also still capable of agglutinating chicken red cells.

The pneumococcal filtrate, diluted 1/5 was then incubated at room temperature with an equal volume of 7.5% chicken red-cell suspension for 1½ hours after which the cells were washed twice, resuspended in 0.75% concentration and tested for agglutinability. They were found to be no longer agglutinated by PR8 virus. Cholinesterase from human red cells had no such effect upon the agglutinability of chicken red cells.

According to Avery and Cullen[6] pneumococcal esterase suffers progressive loss of activity at temperatures above 50° C and becomes completely inactive within 10 minutes at 70° C. McCrea has reported[10] similar progressive destruction between 56° C and 65° C of the substance in normal rabbit serum which inhibits influenzal hemagglutination and complete inactivation when such serum was heated for 30 minutes at any higher temperatures. Had we been able to confirm McCrea's observations, a striking analogy could have been established between the inhibiting agent and the cholinesterases studied. As Table III shows, however, the inhibitory power of normal guinea pig and rabbit sera were found to be either unaffected or were somewhat enhanced by temperatures between 60° C and 70° C. Type V pneumococcal filtrate and human red-cell cholinesterase, on the other hand, were entirely inactive after being heated to 60° C for half an hour.

It is interesting to note (Table IV) that when the inhibition test is performed at room temperature agglutination may occur at first

10 McCrea, J. F., *Australian J. Exp. Biol and Med. Sci.*, 1946, **24**, 283.

in tubes in which with further incubation the clumps will be dispersed under the influence of the inhibiting agent. Whether the agent is a serum or a pneumococcal preparation, it appears to be slower in its action at this temperature than the agglutinating factor but in time is capable within its effective concentration of counteracting it completely.

Discussion. Tables I to IV, inclusive, reveal significant similarities in the action of pneumococcal filtrates, human red-cell extracts, and sera of 3 species of non-immunized animals. Since chicken red cells exposed to the Type V pneumococcal preparation rendered them refractory to agglutination by PR8 virus whereas the human red-cell extract failed to produce any such effect, some doubt is aroused regarding the relationship between specific cholinesterase (which the human cell extract contains) and the inhibitory phenomenon.

When these discordant findings have been considered, however, the fact remains that all of the agents employed in this study were capable of inhibiting influenzal haemagglutination and all contained either specific or non-specific cholinesterase.

Filtrates of a number of other organisms were tested for the inhibitory effect, but all were found to be inactive. The organisms tried included *Staph. aureus, Strept pyogenes, Strept. mitior, C. diphtheriae, C. hoffmanni, E. coli, B. subtilis,* and *Prot. vulgaris.* All of

TABLE III.
Agglutination-inhibition Titers.

Inhibiting agents	Treatment			
	Unheated	60°C ½ hr	65°C* ½ hr	70°C* ½ hr
Type V pneumococcus filtrate	1/256	<1/4		
Red-cell cholinesterase	1/32	<1/4		
Guinea pig serum	1/320	1/1280	1/1280	1/1280
Rabbit serum	1/320	1/320	1/1280	1/2560

* Sera were diluted 1/10 with physiological saline prior to heating.

these organisms have been reported[11] to contain no cholinesterase.

Summary. 1. Filtrates and autolysates of Type I and Type V pneumococcus cultures inhibited the agglutination of chicken red cells by influenza virus. 2. Chicken red cells treated with such a filtrate (or autolysate) could not then be agglutinated by the virus. 3. Human red-cell extracts and normal sera of man, guinea pigs, and rabbits were also shown to be capable of inhibiting agglutination of chicken red cells by influenza virus, although some differences of behavior were noted. 4. The presence of a cholinesterase in each of these agents was shown and their possible significance in agglutinin inhibition was discussed.

11 Vincent, D., and de Trat, J., *Compt. Rend. Soc. Biol.,* 1945, **139**, 1148.

TABLE IV.
Inhibition Titers.

Incubation time	G.P. serum vs PR8 (Type A)	G.P. serum vs Lee (Type B)	Rabbit serum vs PR8 (Type A)	Rabbit serum vs Lee (Type B)	Type V pneumo filtrate vs PR8
25 min	<1/10	1/10	0	1/20	1/10
90 min	1/1280	1/2560	1/640	1/80	1/80
16 hr*	1/2560	1/2560	1/1280	1/1280	1/160

* Placed in 4°C refrigerator after 90 minutes at indicated temperature.

15970 P

Ovarian Cholesterol Levels During the Reproductive Cycle of the Rat.*

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Evidence that ovarian cholesterol may be a precursor of the ovarian hormones, particularly progesterone, has been by inference, both positive and negative. Comparison of results from previous studies on cholesterol variations during the ovarian cycle is difficult because of the lack of uniformity in presenting data, *i.e.*, some report the free cholesterol and others only the cholesterol esters. By histochemical methods, no changes were observed in ovarian cholesterol during the reproductive cycle in humans[1] although chemical analysis of human corpora lutea indicated a significant decrease in cholesterol esters only in the actively functioning corpus luteum of pregnancy.[2] In the pig, the cholesterol ester content of the corpora lutea was found to vary inversely with the activity of the gland.[3] Boyd[4] found that in the whole ovary of the rabbit, there was an increase in the free cholesterol during the first half of pregnancy followed by a decline while the cholesterol esters increased only during the last half of pregnancy. These results were interpreted as indicating that the rabbit corpora lutea reach a peak of activity about the middle of gestation. No change in free cholesterol was observed in the ovaries of guinea pigs during the course of gestation.[5] Based on histochemical studies of the corpora lutea of diestrum in the rat, Everett[6] strongly suggested that cholesterol serves as a precursor of progesterone.

A systematic examination of cholesterol in the ovary of the rat has been undertaken in this laboratory, and this report will show the changes in cholesterol level of the ovary in diestrum, pseudopregnancy, and at several intervals during pregnancy and lactation.

Methods. Total cholesterol was determined by the method of Chamberlain[7] which consists essentially in saponification of the lipids in alkaline alcohol, extraction of the lipids with ether, and determination of the cholesterol directly on the washed ether-extract residue by the Liebermann-Burchard reaction. A Bausch and Lomb colorimeter was used for color comparison. This procedure gave results which were comparable with those obtained by the digitonin precipitation method[8] both in recoveries of pure cholesterol from solution as well as from pooled ovarian tissue. For example, the average value of 3 determinations on an ovarian tissue sample yielded 0.39 mg by the digitonin method as compared to 0.37 mg for aliquot samples using the Chamberlain method.

The stages of the reproductive cycle at which the ovaries were analyzed are given in the table. Pseudopregnancy was induced by electrical stimulation of the cervix. The ovaries were trimmed of extraneous tissue under the binocular dissecting microscope and weighed to the nearest 0.1 mg.

Results. The cholesterol values are best compared on the basis of parts per thousand. It is noted in Table I that the highest levels were found on the 8th day of pseudopregnancy, 10th day of pregnancy and the 10th day of lactation. The cholesterol in the ovaries on the 15th day of lactation, while not as high as obtained above, nevertheless was significantly higher than that found at diestrum (P value between .05 - .02). The highest levels of cholesterol were found only at a time when it

1 Kaufman, C., and Raeth, K., *Arch. f. Gynak.*, 1927, **130**, 128.

2 Weinhouse, S., and Brewer, J. I., *J. Biol. Chem.*, 1942, **143**, 617.

3 Bloor, W. R., Okey, R., and Corner, G. W., *J. Biol. Chem.*, 1930, **86**, 291.

4 Boyd, E. M., *J. Biol. Chem.*, 1935, **108**, 610.

5 Boyd, E. M., *J. Biol. Chem.*, 1936, **112**, 591.

6 Everett, J. W., *Am. J. Anat.*, 1945, **77**, 293.

7 Chamberlain, E., *J. Physiol.*, 1929, **66**, 249.

8 Kelsey, F. E., *J. Biol. Chem.*, 1939, **127**, 15.

TABLE I.
Cholesterol in Rat Ovaries During the Reproductive Cycle.*

	Period (days)	No. of rats	Body wt g	Ovarian wt mg	Total cholesterol mg	Cholesterol parts/1000	No. of fetuses or suckling young
Control	diestr.	10	223	62.5 (36.3- 94.8)	.45 (.30-.65)	7.3 (5.0- 9.0)	—
Pregnancy	10	7	223	60.9 (40.5- 66.9)	.64 (.53-.74)	10.6 (8.6-13.1)	7
	14	9	259	83.5 (73.5- 99.8)	.63 (.44-.79)	7.6 (4.8- 9.8)	9
	18	5	331	104.8 (100.0-105.2)	.80 (.72-.93)	7.7 (7.0- 9.3)	12
Pseudopreg.	8	6	218	43.5 (35.8- 53.8)	.47 (.36-.58)	11.0 (7.7-13.7)	—
Lactation	1	5	224	67.1 (60.0- 77.2)	.49 (.46-.51)	7.3 (6.4- 8.5)	9
	5	6	223	58.4 (41.4- 78.7)	.41 (.30-.51)	7.2 (5.1- 9.7)	8
	10	7	220	49.9 (42.3- 58.2)	.54 (.36-.68)	11.4 (6.2-14.3)	8
	15	7	245	52.0 (34.6- 71.3)	.44 (.30-.52)	8.6 (7.7- 9.7)	8

* All values are average, the range indicated by ( ).

is possible to demonstrate placentomata in the uterus and thus when there must be large amounts of circulating progesterone.[9] It is possible to demonstrate placentomata in the uterus on the 15th day of lactation provided an adequate number of young are suckling (8 or more).

The total ovarian cholesterol was found to be highest on the 18th day of pregnancy yet it is impossible to secure placentomata in the

non-pregnant horn of a unilateral pregnant rat at this time.[9] Since functional corpora lutea or progesterone in the rat are necessary for the maintenance of pregnancy, it seemed odd that the cholesterol content of the ovaries in parts per thousand did not remain high throughout pregnancy. This suggests further studies on the ovaries of pregnant rats. It cannot be stated conclusively from these results that cholesterol acts a precursor of progesterone but in certain instances it appears that high ovarian cholesterol is associated with high levels of progesterone secretion.

[9] Burrows, H., *Biological Actions of Sex Hormones*, Cambridge Univ. Press, 1945, 419.

15971

Turnover of Serum Protein in Adrenalectomized Rats.*

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In previous experiments adrenal cortical activity was found to have no effect on the

concentration of serum antibodies or on any other fraction of the serum proteins.[1] This finding, however, did not preclude the pos-
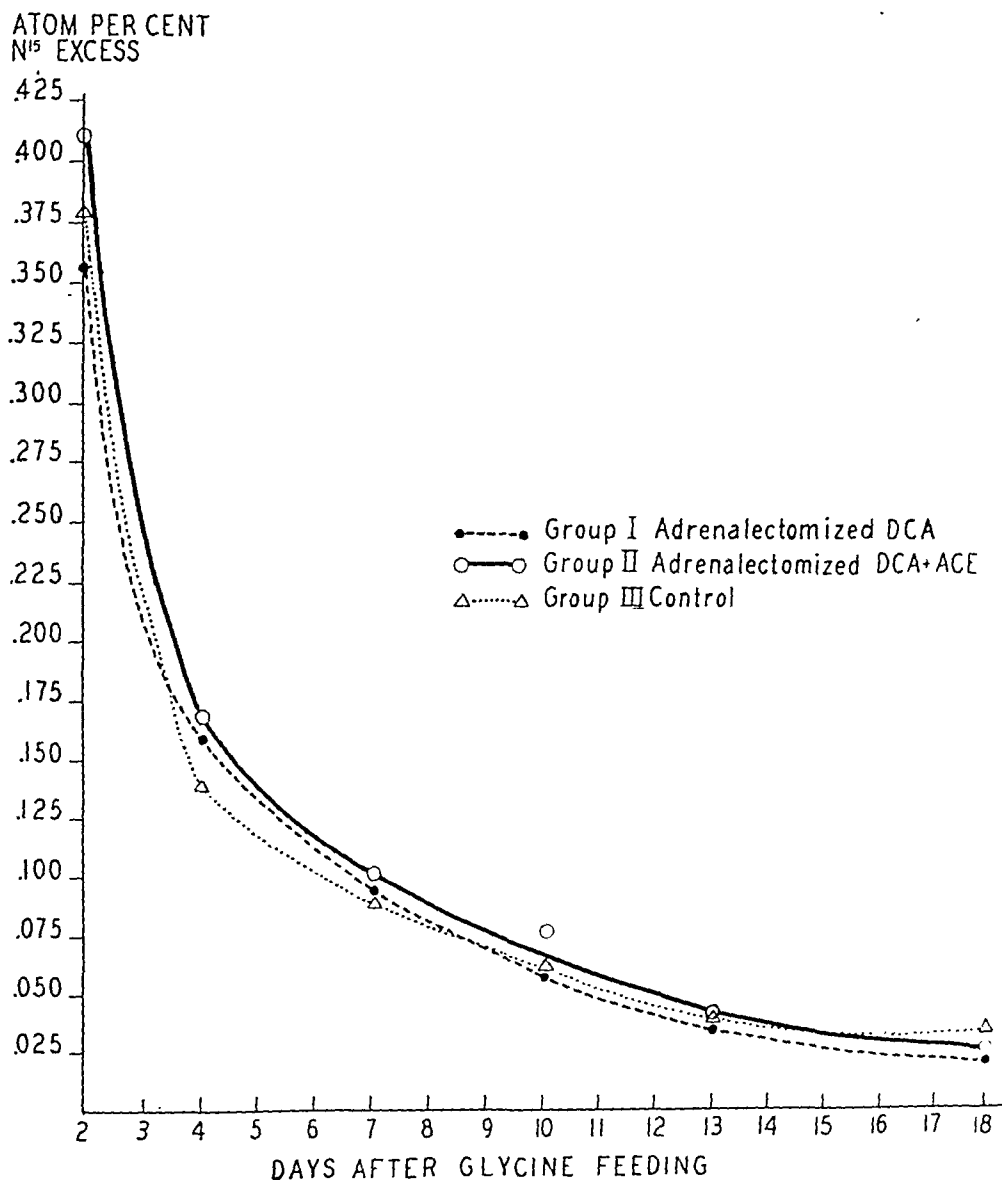
Fig. 1.

The half lifetime of serum proteins, estimated from the rate of decline of atom % N^{15} excess, is the same in all 3 groups.

sibility that the adrenal cortical hormones exerted a considerable influence on the turnover of the serum proteins. It therefore seemed desirable to determine the rates of synthesis and degradation of the serum proteins in adrenalectomized rats, particularly in view of the widely held belief that adrenal cortical

activity influences protein catabolism.[2]

Methods and materials. Nine adult male

[1] Eisen, H. N., Mayer, M. M., Moore, D. H., Tarr, R. R., and Stoerk, H. C., PROC. SOC. EXP. BIOL. AND MED., 1947, 65, 301.

[2] Long, C. N. H., Katzin, B., and Fry, E. G., *Endocrinology*, 1940, 26, 309.

TABLE I.
Rate of Decline of Atom % N^{15} Excess in Total Serum Proteins.

Group	Procedure	Animal No.	Days after conclusion of feeding isotopically-labelled glycine					
			2	4	7	10	13	18
I	Adrenalectomy	58	—	.162	.093	.059	.038	.018
	DCA	61	.345	.155	.091	.052	.030	.013
		67	.368	.167	.105	.064	.038	.033
II	Adrenalectomy	68	.343	.200	.134	.068	.051	.033
	DCA	73	.473	.159	.092	.065	.044	.024
	ACE	65	.420	.151	.105	.084	.034	.017
III	Controls	74	.399	.142	.107	.072	.049	.033
		75	.353	—	.083	.065	.038	.031
		69	.381	.145	.081	.049	.038	.029

albino rats of the Sherman strain, ranging in weight from 250 to 295 g were divided into 3 groups. The animals of Group I were bilaterally adrenalectomized under ether anaesthesia and were maintained during the postoperative period on Rockland rat diet, subcutaneous injections of desoxycorticosterone acetate (DCA),§ 0.4 mg on alternate days, and on drinking water containing 1% sodium chloride. The animals of Group II were treated similarly. However, from the ninth postoperative day to the end of the experiment they received, in addition, daily injections of 0.5 ml adrenal cortical lipoextract (ACE).ǁ Group III was composed of unoperated, uninjected control animals.

From the tenth to the twelfth post-operative days animals in all three groups were fed glycine tagged with heavy nitrogen. The isotopically labelled glycine was incorporated into the diet and administered so that each animal received 0.4 mg per gram body weight. Beginning 48 hours after the cessation of the glycine feeding, and repeated at intervals of from 2 to 10 days, 0.5 ml of blood was collected from a freshly cut surface of the tail and the total serum proteins were examined for their content of N^{15} by methods previously described[3]. After 30 days the animals were exsanguinated and their blood analyzed for total protein, albumin, globulin, and total non-protein nitrogen.¶

Results. The animals of all 3 groups gained approximately 10% in body weight. The serum total proteins, albumin, globulin, and non-protein nitrogen values determined at the end of the experiment (40th post-operative day) were the same in Group I and in Group II and were within normal limits. The heavy nitrogen contents of the serum proteins are given in Table I and in Figure 1.

Discussion. Following the feeding of labelled glycine, the N^{15} which had been incorporated into the serum proteins was replaced by N^{14} at a rate which was identical for all 3 groups. These data indicate that the rate of synthesis of serum proteins is not affected by adrenalectomy or by the administration of ACE to adrenalectomized rats. Since the total "pool" of serum proteins seems to have been approximately the same in the several groups, the rate of serum protein breakdown appears likewise to have been no different. These data do not exclude the possibility that adrenal cortical activity exerts an influence on the turnover of *tissue* proteins.

The half lifetime of serum proteins in the rat (1½ days) was found to be considerably briefer than the value (about 12 days) found in rabbits by Schoenheimer et al.[4] Unless the

§ Generously provided by Schering Corporation, Bloomfield, N. J.

ǁ Upjohn Company, Kalamazoo, Mich.

[3] Rittenberg, D., Keston, A. S., Rosebury, F., and Schoenheimer, R., *J. Biol. Chem.*, 1939, 127, 291.

¶ We wish to thank Miss Genevive Corbett for these determinations.

[4] Schoenheimer, R., Ratner, S., Rittenberg, D., and Heidelberger, M., *J. Biol. Chem.*, 1942, 144, 545.

repeated bleedings exerted a more profound effect on rats than on rabbits, no explanation for this difference is apparent.

Summary. The turnover of serum protein in the rat is unaffected by adrenalectomy, or by the administration of adrenal cortical lipo-extract (ACE) to adrenalectomized animals.

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Increased Dental Caries Activity in the Syrian Hamster Following Desalivation.*

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The Syrian hamster (*Cricetus auratus*) has been used in the study of experimental dental caries and appears well suited for such investigation since the disease process in this animal appears similar to that described in humans.[1] Furthermore, the administration of fluorine with a caries producing diet has been shown to reduce the caries incidence,[2] a finding which roughly parallels observations in man.[3] It appeared of some importance to determine whether removal of the major salivary glands would be accompanied by increased caries activity since it is well known that removal, agenesis, or greatly reduced function of the salivary glands in humans is accompanied by rampant decay.[4-5] Such a response might be expected since increased caries activity subsequent to salivary gland extirpation has been observed in the albino rat.[6-9]

Experimental. The animals were of pure inbred stock and were raised on Purina Rabbit Checkers plus weekly supplements of fresh vegetables. They were kept in metal cages with wood shavings for bedding. When 42 days old ($\pm$ 4 days), the animals were separated into a control group (Group I) and an experimental group (Group II). A comparable supplementary group (Group Ia) was available for additional comparisons. The experimental animals were desalivated during a period of 2 weeks. The parotid, submaxillary, and major sublingual glands were removed, under ether anesthesia, through a single midline incision beginning about 5mm from the mandibular symphysis and extending to the manubrium. Control animals were not operated upon. One week after the last operation,[‡] an experimental diet and tap water were provided *ad libitum* to Groups I and II. The diet consisted of whole wheat flour, 40%; whole powdered milk, 30%; glucose, C.P., 20%; alfalfa, 5% brewers yeast, 4%; and *sodium chloride, 1%*. The supplementary control animals (Group Ia), which were used for another study, were of the same age but had been started on the diet 25 days previously.

Animals were weighed at weekly intervals.

* This work was supported, in part, by a grant from the Eastman Dental Dispensary of Rochester, N. Y.

† Now at the Harvard School of Dental Medicine, Boston, Mass.

1 Keyes, P. H., *J. D. Res.*, 1946, **25**, 341.

2 Dale, P. P., Lazansky, J. P., and Keyes, P. H., *J. D. Res.*, 1944, **23**, 445.

3 Deatherage, C. F., *J. D. Res.*, 1943, **22**, 173.

4 Prinz, H., *D. Cosmos*, 1932, **74**, 129.

5 Faber, M., *Acta. Paediat.*, 1942, **30**, 148.

6 Kondo, S., Ichikawa, T., and Arai, M., *Tr. Soc. path. jap.*, 1938, **28**, 461.

7 Hukusima, M., *Tr. Soc. path. jap.*, 1940, **30**, 245.

8 Cheyne, V. D., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 587.

9 Weisberger, D., Nelson, C. T., and Boyle, P. E., *Am. J. Orthodontics and Oral Surg.*, 1940, **26**, 88.

‡ Four animals failed to survive the operation and, consequently, have not been included in the data.
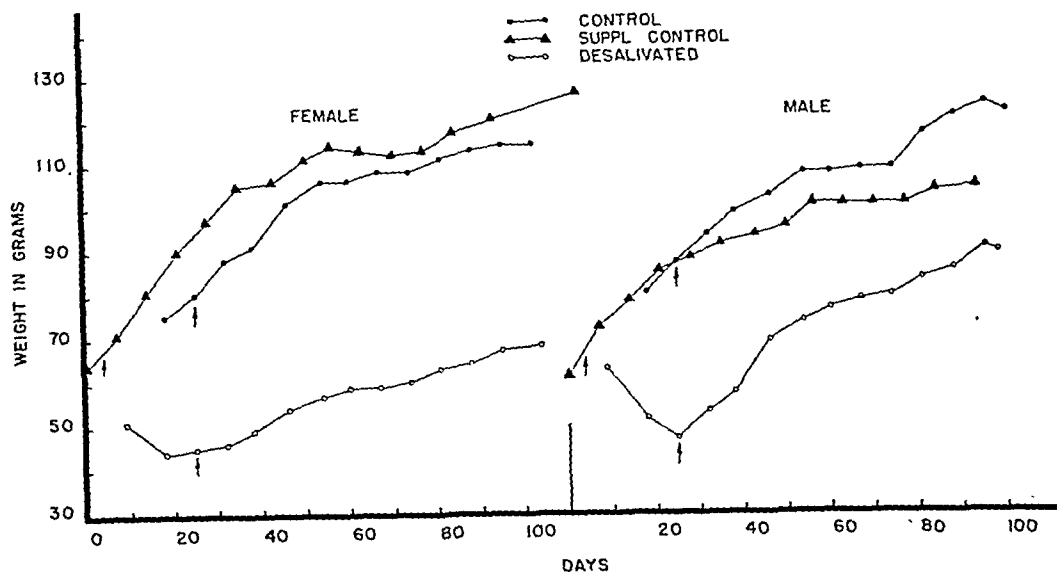
Fig. 1.
Average weight curves for the 3 groups. Arrows indicate the time at which the experimental diet was begun.

Group I and II animals were sacrificed over a 6 day period starting 73 days after the experimental diet had begun. An autopsy was done on each of these animals. Male animals in the supplementary group were killed after 93 days on the diet and female animals after 111 days. Heads were preserved in 10% formaldehyde, stripped of soft tissue, and scored;[10] the number of carious teeth (M. A.), the number of cavities (C. N.), and the total score (T. S.) were recorded. Total score (T. S.) is an arbitrary figure which represents the amount of tooth structure destroyed. All teeth were scored by the same investigator. Data independently obtained for Group Ia animals by the co-author were in excellent agreement.

Differences among the means of average M. A., average C. N., and average T. S. for each of the groups compared were analyzed by the Student-t method.[11] Comparison was made between Group Ia ♂ ♂ and ♀ ♀; Group Ia and I ♂ ♂; Group Ia and I ♀ ♀; Group I and II ♂ ♂; and Group I and II ♀ ♀. An arbitrary level of significance of p = .01 was chosen; all results less than this value were

considered significant.

Differences among the groups were further compared by the calculation of "percentage of molars affected" and "average percentage of tooth destroyed" for each of the several types of molars. This method of calculation is illustrated beneath Table III.

Results. Average weight curves for the 3 groups are presented in Fig. 1. Desalivated animals lost weight following the operation and remained lighter than those of the control groups. Desalivated females were especially uniform in their weight response. Control animals were sleek, fat, and healthy in appearance. The experimental animals were noticeably emaciated, were more active, and showed at autopsy, a pronounced reduction in depot fat.

Inspection of the mouths prior to administration of the experimental diet disclosed a generalized dryness of the oral tissues in the case of the operated animals whereas the mucous membranes of Group I animals were bathed by copious amounts of salivary secretion.

Caries scores and statistical findings are presented in Tables I and II. *Control groups.* Despite the fact that Group Ia males had been maintained on the experimental diet

[10] Keyes, P. H., *J. D. Res.*, 1944, **23**, 439.
[11] Fisher, R. A., *Statistical Methods for Research Workers*, 7th Ed., 1938, Chapt. 5.

TABLE I.
Effect of Desalivation on Caries Activity.

Group	Male Animal No.	Male Max. + Mand. scores M.A.	C.N.	T.S.	Female Animal No.	Female Max. + Mand. scores M.A.	C.N.	T.S.
Suppl. control	27	9	11	18	42	1	1	¼
	82	9	10	31	12	3	3	1
	96	5	5	34	15	6	8	5
	69	10	12	59	57	5	6	7
	89	12	14	77	34	6	6	8
	32	11	11	104	75	6	6	9¼
	104	12	14	107	98	6	7	15
	51	12	14	132	122	8	9	29
					50	9	11	64
Avg		10	11	70		6	6	16
Control	123[1]	5	5	2¼	71[3]	1	1	½
	112[2]	6	6	11	46	4	5	2
	124	12	12	73	100	8	8	13
	13	11	15	111	88[4]	4	4	13
					63[5]	7	8	29
Avg		9	10	49		5	5	12
Experimental	122×[1]	11	15	72	68[3]	12	12	208
	113[2]	12	14	166	×	12	13	239
	92	12	15	221	93[4]	12	12	257
					64[5]	12	12	278
Avg		12	15	153		12	12	246

M.A. = Molars affected.
C.N. = Cavity number.
T.S. = Total score.
Total scores of 138 and 144 indicate complete destruction of maxillary and mandibular molar teeth, respectively.
Animals indicated by same superscript are litter mates.

for 18 days less than the females, the average M. A., C. N., and T. S. were higher for male animals than for female. This difference was statistically significant. Differences between Groups Ia and I were not significant. *Experimental group.* The incidence of dental caries in both male and female animals was increased by desalivation. Average values for M. A., C. N., and T. S. are in every case higher for desalivated animals than for controls. Differences among the male hamsters (Groups I and II) were not statistically significant; *differences among famales of the same groups were highly significant.* Several factors may have obscured statistical differences among the male animals; (1) high caries incidence among unoperated animals, (2) greater variability of response in the male and (3) the small number of animals employed. It is interesting to note that although male animals ordinarily accumulate more dental

decay, females experience a greater amount of tooth destruction subsequent to desalivation.

Considerable variation was observed among individual molar types with respect to percentage of teeth decayed and average percentage of tooth destroyed (Table III). However, in every case but one (♂, Group II, mand. 3rd molars) both measures of caries activity were greater for the desalivated group than for either control group. Every tooth of the experimental animals with one exception (as cited above) showed evidence of dental caries, while in several instances complete or almost complete destruction of all crowns was recorded, e. g. ♀, Group II, max. molars.

Discussion. Under the conditions of this experiment, desalivation of hamsters was followed by a *definite reduction in body weight* especially in the female animals. Decreased food consumption may have contributed to this

TABLE II.
Statistical Evaluation of Caries Scores.

Comparison			Avg	t	p
Group Ia	♂♂ vs. ♀♀		M.A.	3.80	.002
			C.N.	3.50	.004
			T.S.	3.40	.004
♂♂	'' '' vs. Group I		M.A.	0.77	.45
			C.N.	0.73	.50
			T.S.	0.70	.50
♀♀	'' '' vs. '' I		M.A.	0.55	.60
			C.N.	0.67	.50
			T.S.	0.46	.65
♂♂	'' I vs. '' II		M.A.	1.73	.14
			C.N.	2.10	.09
			T.S.	2.05	.10
♀♀	'' I vs. '' II		M.A.	5.90	.0006
			C.N.	5.27	.001
			T.S.	14.9	<.0002

$$t = \sqrt{\frac{m_1 - m_2}{\sigma^2_{m_1} + \sigma^2_{m_2}}}$$

where m_1 and m_2 = means compared

σ_{m_1} and σ_{m_2} = unbiased estimate of the standard deviation of the means

$$\sigma_m = \frac{\sigma}{\sqrt{N}}$$

σ = unbiased estimate of standard deviation

N = No. of animals

$(N_1 + N_2 - 2)$ = Degrees of freedom in t

TABLE III.
Caries Incidence as Found in Individual Molars.

	Molar teeth	Group Ia (Suppl. control)		Group I (Control)		Group II (Experimental)	
		% molars affected	Avg % of tooth decayed	% molars affected	Avg % of tooth decayed	% molars affected	Avg % of tooth decayed
Max.	Female						
	1st	60.	1.6	40.	0.56	100.	97.
	2nd	40.	3.0	50.	8.4	100.	100.
	3rd	30.	2.2	00.	0.0	100.	100.
Mand.	1	70.	7.0	80.	2.3	100.	93.
	2	60.	17.	60.	12.	100.	90.
	3	30.	1.3	10.	0.10	100.	55.
Max.	Male						
	1	90.	7.8	80.	2.0	100.	53.
	2	70.	21.	40.	13.	100.	61.
	3	80.	28.	80.	14.	100.	54.
Mand.	1	90.	40.	90.	16.	100.	68.
	2	90.	34.	100.	49.	100.	75.
	3	80.	21.	50.	15.	80.	13.

Sample calculation (Maxillary 1st molar, Group Ia, female):

Percentage of molars affected =
$$\frac{\text{No. of carious max. 1st molars}}{\text{Total No. of max. 1st molars}} \times 100 = \frac{11}{18} \times 100 = 60.\%$$

Avg percentage of tooth destroyed =
$$\frac{\text{Sum of total scores of all carious max. 1st molars}}{\text{Sum of available total scores of all max. 1st molars}} \times 100 = \frac{7.75}{486} \times 100 = 1.6\%$$

effect for several measurements on group food intakes revealed a consistent reduction in the desalivated group. The effect of the operation, per se, cannot be stated since a sham operation was not performed on either of the control groups. Weight loss following operation has been observed in the rat.[12] Reports on the subsequent changes in weight after recovery of rats from comparable operations are not in agreement. Hukusima[7] found a reduction in body weight; data presented by Cheyne[12] showed an appreciable reduction in 5 of the 8 groups studied although the animals remained in good health. Weisberger et al.[9] reported no apparent ill effects on subsequent growth and development.

The higher incidence of decay observed in male hamsters (Group Ia) is in agreement with previous reports.[1,13-16]

Since Group Ia was on the experimental regimen for appreciably longer periods of time than Group I, a higher incidence of decay would be expected in the former. This proved to be true although the differences were not large and were not shown to be significant. This finding tended to increase the number of control observations and strengthened the argument that changes observed in desalivated animals could not be explained on the basis of normal variation.

The increased caries incidence following salivary gland extirpation in the hamster conforms with observations made in the rat and in man. It is difficult to compare the response of the rat and hamster because of variable experimental conditions and dissimilar scoring technics. In the rat, there is a rise in number of animals[6-7] and number of teeth[8] affected as well as an increase in the number of carious lesions.[8] With the hamster, large differences are noted only in the amount of tooth destruction, especially in the female. The rapidity of the carious

process may have obscured an increase in number of cavities formed.

The mechanism of increased caries activity cannot be explained by the present findings. Two possibilities suggest themselves: alteration in systemic factors and/or alteration in local factors. The latter explanation is probably more correct. Interference with salivary flow (as observed) would reduce the normal cleansing action of the saliva. In addition, it is not unlikely that the saliva of these rodents contains antibacterial agents and other compounds similar to those observed in human saliva, which are known to inhibit the growth[17] or oppose the action of oral bacteria.[18-19]

Summary and conclusions. 1. Seven hamsters (3 ♂ ♂, 4 ♀ ♀) were desalivated and compared with a control group (4 ♂ ♂, 5 ♀ ♀) and a supplementary control group (8 ♂ ♂, 9 ♀ ♀). It has been found that the animal can be desalivated easily and that it will usually survive operations.

2. Under the experimental conditions, extirpation of the glands resulted in a sharp reduction in body weight. Subsequently, body weight remained below that of the control groups.

3. Dental caries activity following operation was increased in both male and female hamsters although the difference was not shown to be statistically significant in the case of male animals. It is probable that a comparable response does occur in males but that a larger group of animals would be necessary for an unequivocal demonstration. Response following desalivation was similar to that reported previously for albino rats and humans.

4. Unoperated male animals experienced more dental decay than did unoperated female animals.

12 Cheyne, V. D., *Thesis, Univ. of Rochester,* 1940, pp. 44, 143, 158, 223, 228.

13 Arnold, F. A., Jr., *Pub. Health Rep.,* 1942, 57, 1599.

14 Dale, P. P., and Keyes, P. H., *J. D. Res.,* 1945, 24, 194.

15 Orland, F. J., *J. D. Res.,* 1946, 25, 445.

16 Keyes, P. H., *J. D. Res.,* 1946, 25, 469.

17 Bibby, B. G., Hine, M. K., and Clough, O. W., *J. A. D. A.,* 1938, 25, 1290.

18 Stephan, R. M., *Science,* 1940, 92, 578.

19 Kesel, R. G., O'Donnell, J. F., Kirch, E. R., and Wach, E. C., *J. A. D. A.,* 1946, 33, 695.

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Observations on the Conversion of Prothrombin to Thrombin.

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Evidence from several sources has indicated that the rate of formation of thrombin in plasma upon the addition of a given thromboplastin is affected by some factor besides the concentration of the prothrombin itself. Seegers, Loomis and Vandenbelt[1] found that thrombin was formed more slowly from very highly purified prothrombin than from prothrombin naturally contained in plasma. In studies of patients who had cirrhosis[2] or pernicious anemia,[3] of newborn infants[4] and of normal dogs and rabbits,[5] the concentration of prothrombin in the plasma has been reported to be less when determined by the 2-stage procedure of Warner, Brinkhous and Smith[5] than by the one-stage method of Quick.[6] In Quick's method the time required for conversion of prothrombin to thrombin constitutes part of the observed clotting time. These differences are attributed by the originators of the 2-stage method to an increased rate of conversion of prothrombin in the one-stage technic. On the other hand Quick[6] thought the differences were due to incomplete conversion of prothrombin in the high dilution required by the 2-stage method. However, when the plasma of patients treated with dicumarol[7] or normal plasma after storage[8] are studied by both methods the 2-stage procedure gives the higher values. Quick[9] has presented evidence that the increase in prothrombin time of plasma after storage is due to the disappearance of a substance termed "prothrombin A" which remains in fresh plasma depleted of prothrombin by treatment of patients or animals with dicumarol or addition of aluminum hydroxide to the plasma. Fantl and Nance[10] also found that plasma freed of prothrombin by treatment with barium carbonate still contained a substance which accelerated the conversion of prothrombin to thrombin. Because in blood coagulation the rate of thrombin formation is more important than the total amount of available prothrombin, such a factor or factors affecting conversion appear to deserve further study.

Method. The prothrombin time of fresh or stored (one to 2 months) human plasma was determined by Quick's method as previously described,[11] except that just before addition of thromboplastin, 0.1 ml of a 0.9% solution of sodium chloride, or whatever material was being tested for action on the conversion rate, was added. Thus, the volume of the clotting system was increased from 0.3 to 0.4 ml.

Standard thromboplastin was prepared by Quick's original method. With this preparation, normal plasma has a prothrombin time of from 17 to 19 seconds. Fresh tissue thromboplastin was made by macerating fresh or frozen rabbit brain with twice its weight of ice-cold normal saline solution. A commercial preparation of acetone-dehydrated rabbit brain* was used. Thromboplastin was prepared from human placenta by the method

1 Seegers, W., Loomis, E., and Vandenbelt, J., *Arch. Biochem.*, 1945, 6, 85.

2 Ziffren, S., Owen, C., Warner, E., and Peterson, F., *Surg., Gynec. and Obst.*, 1942, 74, 463.

3 Warner, E., and Owen, C., *Am. J. M. Sc.*, 1942, 203, 187.

4 Owen, C., Hoffman, G., Ziffren, S., and Smith, H., Proc. Soc. Exp. Biol. and Med., 1939, 41, 181.

5 Warner, E., Brinkhous, K., and Smith, H., *Am. J. Physiol.*, 1939, 125, 296.

6 Quick, A., *The Hemorrhagic Diseases and the Physiology of Hemostasis*, Springfield, Ill., Charles C Thomas, 1942, pp. 36 and 40.

7 Hurn, M., Barker, N., and Mann, F., *Am. J. Clin. Path.*, 1947, 17, 712.

8 Lord, J., and Pastore, J., *J. A. M. A.*, 1939, 113, 2231.

9 Quick, A., *Am. J. Physiol.*, 1943, 140, 212.

10 Fantl, P., and Nance, M., *Nature*, 1946, 158, 708.

11 Hurn, M., Barker, N., and Magath, T., *J. Lab. and Clin. Med.*, 1945, 30, 432.

of Zondek and Finkelstein.[12]

The materials tested for prothrombin-converting activity were plasma and serum from human beings and crude platelet extract. The platelet extract was prepared as follows. Oxalated plasma was separated from the cells by gentle centrifugation; then the suspension of platelets thus obtained was centrifuged for 30 minutes at 3,000 revolutions per minute. The plasma was carefully drained from the tubes, after which the sticky pellet of sediment was removed with a stirring rod. The sediment, which thus contained a minimal amount of plasma, was triturated in a mortar with 0.9% solution of sodium chloride containing 0.001 molar sodium oxalate. One milliliter of this fluid was used for the amount of sediment obtained from 16 ml of plasma. Serum was used only after it had stood 3 to 4 hours at room temperature to allow for destruction of thrombin. Neither the platelet extracts nor the serum used caused clotting when mixed with oxalated plasma. The plasma tested was drawn and oxalated as for a prothrombin determination or in some experiments the silicone technic of Jaques and his associates[13] was used. When the silicone technic was used all pipets and tubes used in handling the plasma throughout the procedure were coated with silicone.

Results. Fresh plasma, serum and platelet extracts were all found to reduce the prothrombin time of stored plasma markedly (Tables I, II and III). The fresh plasma was so highly diluted that little of the effect could be attributed to its content of prothrombin; the same was true of platelet extract. Platelet extract showed considerably more prothrombin-converting activity than did serum or plasma. When the thromboplastin of rabbit brain was replaced with normal saline solution the clotting time was greatly prolonged, showing that the platelet extract potentiated the action of the tissue thromboplastin. Platelet

* Obtained from the Difco Laboratories, Inc., Detroit, Mich.

12 Zondek, Bernhard, and Finkelstein, Michael, PROC. SOC. EXP. BIOL. AND MED., 1945, 60, 374.

13 Jaques, L., Fidlar, E., Feldsted, E., and Macdonald, A., *Canad. M. A. J.*, 1946, 55, 26.

TABLE I.

Effect on Stored Plasma with Standard Thromboplastin of Plasma and Serum Drawn from Same Subject at the Same Time.

Material added to plasma	Dilution	Clotting time, sec
Saline		82
Plasma	1:10	36
	1:20	41
	1:40	49
	1:80	57
Serum*	1:10	31
	1:20	37
	1:40	43
	1:80	57

* Undiluted serum usually restored the clotting time to the normal value of 18 seconds.

TABLE II.

Potentiation of Standard Thromboplastin with Platelet Extract.

Plasma	Material added	Thromboplastin	Clotting time, sec
Stored	Saline	Standard	90
	Platelet extract	,,	13
	,, ,,	None	86
	Plasma from which extr. made dil. 1:5	Standard	28
Fresh	Saline	,,	18
	Platelet extr.	,,	13
	,, ,,	None	47
	Saline	Standard dil. 1:50	39
	Platelet extr.	Standard dil. 1:50	23

TABLE III.

Potentiation of Fresh Tissue Thromboplastin with Platelet Extract.

Plasma	Material added	Thromboplastin	Clotting time, sec
Stored	Saline	Fresh tissue	52
	Platelet extr.	,, ,,	15
	,, ,,	None	45
Fresh	Saline	Fresh tissue	20
	Platelet extr.	,, ,,	16
	,, ,,	None	56
	Saline	Fresh tissue, dil. 1:50	32
	Platelet extr.	Fresh tissue, dil. 1:50	23

extract was observed to potentiate the action of thromboplastin from dried rabbit brain, fresh rabbit brain, acetone-dehydrated rabbit brain and human placenta on stored

TABLE IV.
Effect of Plasma Drawn from Same Subject by Various Technics. Standard Thromboplastin. Stored Plasma.

Material added	Dilution	Clotting time, sec
Saline		145
Oxalated plasma	1:10	30
	1:40	49
Oxalated plasma (silicone technic)	1:10	31
	1:40	46
Native plasma* (silicone technic)	1:10	23
	1:40	36

* Tested within 5 minutes after blood drawn; blood clotted in original tube after 30 minutes.

plasma. A similar, but less striking, potentiation between platelet extract and tissue thromboplastin was observed when fresh plasma was used. Thus although the prothrombin-converting activity of fresh plasma is rather great it is still not maximal, especially if dilute thromboplastin is used. However, since the extracts represent 16 times their volume of plasma, it would seem reasonable that the activity of the plasma might not be less than that of its platelets Plasma had the same converting activity when drawn and handled with silicone technic as when exposed to glass (Table IV). Thus it seems unlikely that this activity is a product of processes incident to the drawing of blood. Native plasma drawn with silicone technic appears to have more prothrombin-converting activity than the same plasma after decalcification. As a rule, all materials tested lost about 90% of their prothrombin-converting

activity on standing 24 hours in the icebox. By heating at 56°C for 30 minutes, most of this activity was destroyed.

Comment. No specific term will be applied at present to the substance which potentiates the conversion of prothrombin to thrombin by tissue thromboplastin. This material appears to be the same as Quick's prothrombin A but it is not converted into thrombin since it remains in the serum. By definition, the conversion-favoring substance might reasonably be regarded as part of the thromboplastin complex. Quick[14] has justly emphasized the fact that if any appreciable amount of active thromboplastin were present in the circulation the blood would not be fluid. This material does appear to exist in the circulation and presumably cannot alone bring about formation of thrombin. The prothrombin-converting activity of the plasma, however, may well be a determining factor when the blood is exposed to a minimal amount of tissue thromboplastin, as it probably often is in cases of thrombosis.

Summary. Fresh plasma, serum and platelet extracts contain a material which potentiates the action of tissue thromboplastin on stored plasma. Platelet extract apparently contains a greater degree of this activity than plasma or serum and potentiates the action of tissue thromboplastin on fresh plasma. This factor favoring prothrombin conversion apparently is present in the circulating blood.

14 Quick, A., *The Hemorrhagic Diseases and the Physiology of Hemostasis,* Springfield, Ill., Charles C Thomas, 1942, p. 70.

15974

Chick Growth Factor in Cow Manure. VI. Effect on Hatchability and Storage in Hens.

MAX RUBIN, A. C. GROSCHKE, AND H. R. BIRD. (Introduced by T. C. Byerly.)

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Whitson et al.[1] showed that as the level of *soybean oil meal in the diet of hens increased* from 0 to 40% in increments of 10%, the hatchability of fertile eggs decreased in spite of apparently adequate quantities of the dietary factors known to affect hatchability. There was no other adverse effect. Whitson et al.[2] also showed that there was considerable improvement in hatchability when 8% of dried cow manure was included in a diet containing 30% soybean oil meal. Shortly thereafter, Bird et al.[3] reported that the inclusion of 6% cow (or steer) manure, 10% sardine meal, or 10% dried skimmilk in this diet corrected the detrimental effect on hatchability.

Rubin and Bird[4] prepared concentrates of a factor found in cow manure, which greatly stimulated the growth of chickens fed a diet containing 35 percent of soybean oil meal but no animal protein. It seemed likely that both growth and hatchability were influenced by the same unknown dietary factor in cow manure. To obtain further evidence on this point one of the concentrates which stimulated growth was tested for its effect on hatchability, and the results are reported herewith.

The hens used in part A of this experiment were Rhode Island Reds that were 10 months old when the experiment commenced. As chicks, they were fed diets which contained 2.5 to 4% of fish meal and they had access to grass range until they were 5 months old. During the latter half of the pre-experimental

TABLE I.
Experimental Diets.

Ingredients	Diet 311 %	Diet 312 %
Yellow corn	57.0	78.3
Alfalfa leaf meal	5.0	5.0
Soybean oil meal	30.0	
Sardine fish-meal		10.0
Steamed bone meal	4.2	3.2
Limestone	2.3	2.0
Butyl fermentation solubles (250 µg riboflavin per g)	0.5	0.5
Salt (94% NaCl; 6% MnSO₄)	0.5	0.5
Iodized salt	0.2	0.2
Vit. A and D feeding oil	0.3	0.3

period they were fed diet 311 (Table I).

The 16 hens used in the experiment were selected because of their low hatchability records and were divided on the basis of these records into 2 comparable groups of 8 birds each. Their average hatchability from the start of egg production to 10 months of age was 54%. This ranged between 34 and 67% for individual hens. The object of the careful selection was to minimize the number of birds and hence the quantity of concentrate used. The hens were kept in a laying battery in an air-conditioned room for the duration of the experiment. Fertile eggs for hatchability studies were obtained by artificial insemination with semen from Barred Plymouth Rock males.

Both groups were fed diet 311 except that the diet of one of the groups was supplemented with 0.1% of the acid insoluble fraction of an extract of cow manure (Rubin and Bird[4]). This supplement was known to produce optimum growth in chicks when fed as 0.05% of an all-plant-protein diet containing 35% of soybean oil meal. The experiment was started March 6 and terminated November 12, 1946. After the groups had been on their respective diets for 6 weeks, the diets were reversed. This dietary regimen was

1 Whitson, D., Titus, H. W., and Bird, H. R., *Poultry Sci.*, 1946, **25**, 52.

2 Whitson, D., Titus, H. W., and Bird, H. R., *Poultry Sci.*, 1946, **25**, 143.

3 Bird, H. R., Rubin, M., Whitson, D., and Haynes, S. K., *Poultry Sci.*, 1946, **25**, 285.

4 Rubin, M., and Bird, H. R., *J. Biol. Chem.*, 1946, **163**, 393.
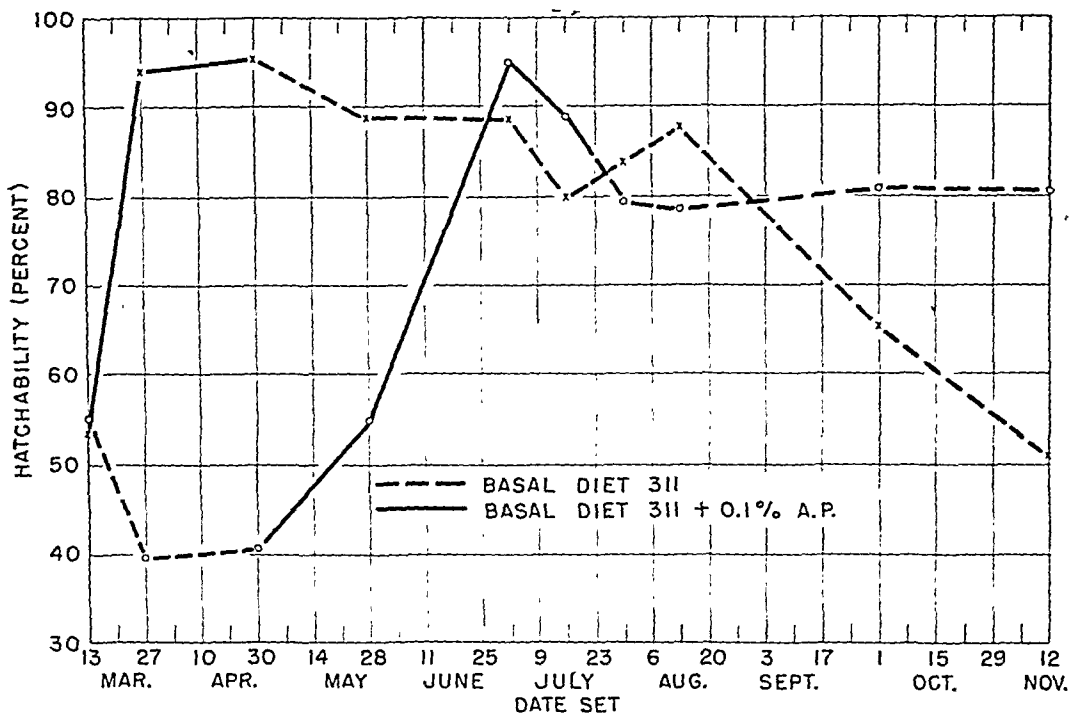
FIG. 1.
Effect upon hatchability of supplementing basal diet 311 with 0.1% of the acid precipitate of the water extract of cow manure.

continued until the seventeenth week of the experiment. At this time and until the termination of the experiment, all the hens were fed diet 311. The dietary changes and the hatchability data are illustrated in Fig. 1.

The concentrate prepared from cow manure brought about a rapid increase in the percentage of hatchable eggs, (Fig. 1), while the hens on the unsupplemented diet continued at a low level of hatchability. When these hens received the concentrate after the initial 6 weeks' period, their percentage of hatchable eggs also increased to a high level. The data in Fig. 1 show that when the supplement was removed from the diet, enough of the hatchability factor had been stored by the hens to enable them to maintain hatchability at a high level for 15 to 16 weeks. The experiment was terminated before the hens in the second group, which had received the concentrate for 11 weeks, had enough time to exhaust their store of the hatchability factor, even though they were fed the diet without the supplement for 16 weeks.

The hens used in part B were crossbreds (Rhode Island Red X Barred Plymouth Rock). During the growing period they had been fed a mash which contained 2 to 3% of fish meal and which supported rapid growth. They had access to grass range during the first 5 months of life. For the following 21 months, until June 4, 1946, they were fed diet 312 (Table I).

On June 4, 45 of these hens were changed to diet 311. They were housed in 2 colony laying houses with New Hampshire males and all eggs with good shells were incubated to determine hatchability. The experiment was terminated August 27, 1946.

During the 5 month period when the hens were fed diet 312 containing fish meal, the average hatchability was 82% of fertile eggs set. Hatchability during the 3 months period in which the hens were fed diet 311 was also 82% (Fig. 2).

Bird *et al.*[5] found that pullets had no

5 Bird, H. R., Rubin, M., and Groschke, A. C., J. Nutrition, 1947, 33, 319.
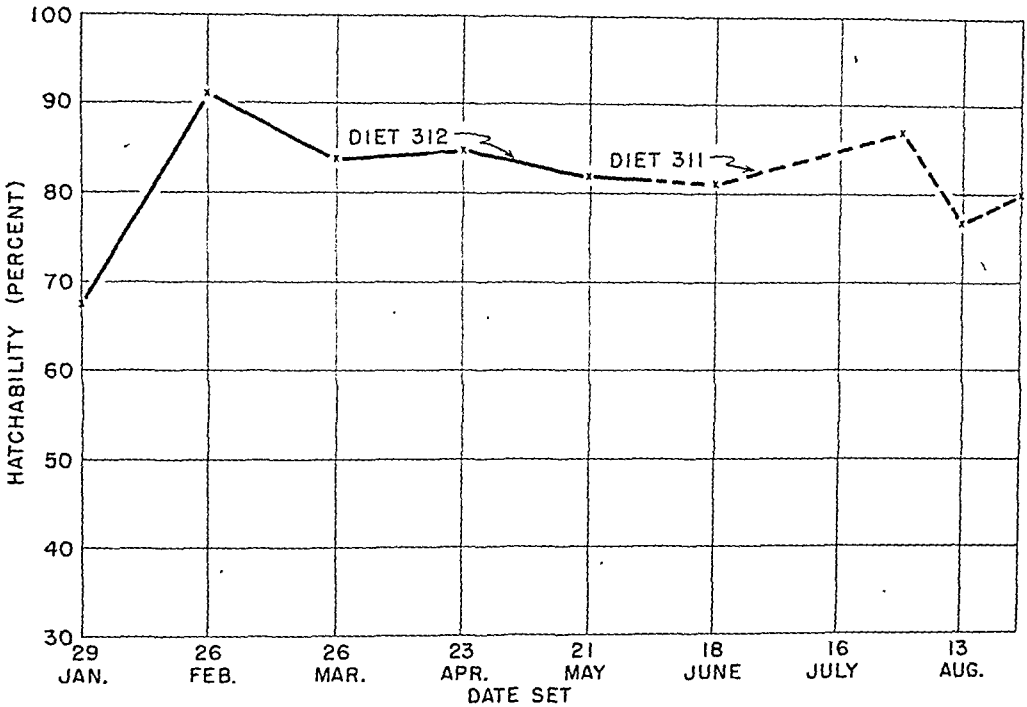
FIG. 2.

Effectiveness of bodily stores in maintaining hatchability in spite of dietary deficiency of the factor found in cow manure and in fish meal.

measurable stores of the hatchability stimulating factor at the time they began to lay. The pullets were of the same breeding as the hens in Part B of this experiment and like them had received a diet containing 2 to 3% of fish meal during the growing period. In this experiment hens fed 10% of fish meal or 0.1 percent of the concentrate from cow manure stored considerable quantities of the factor. Differences in the quantity of intake may account for the different results, or it may be that the mature bird has greater storage capabilities than the growing bird.

Summary. An acid precipitate of a water extract of cow manure which was highly potent as a source of the chick growth factor was shown to possess a high potency of the hatchability factor required by hens fed an all-plant-protein diet. This result is in accord with the view that the same dietary factor influences both growth and hatchability.

Hens that obtained the hatchability factor from the above mentioned concentrate or from fish meal, stored sufficient quantities of the factor, so that high hatchability was maintained for at least 12 to 15 weeks even when a deficient diet was fed.

15975

A Simplified Method for the Quantitative Determinations on Free Pregnanediol Excretion in Pregnancy.

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Venning and Browne[1] described a method for the isolation of sodium pregnanediol glucuronidate in the urine and theorized that this chemical substance represented the end-product in the metabolism of progesterone. Further studies confirmed the relationship of pregnanediol excretion to the luteal phase of the ovarian cycle and to normal pregnancy. Furthermore, a correlation between the administration of progesterone and the excretion of pregnanediol has been established although in the non-pregnant individual only a small fraction of progesterone administered can be recovered as sodium pregnanediol glucuronidate.

The gravimetric method of Venning for the quantitative estimation of pregnanediol is long and tedious and it has the disadvantage that a number of factors other than progesterone influence the excretion of glucuronidate. Astwood and Jones[2] described a method for the determination of free pregnanediol utilizing the hydrolysis of glucuronidate. Talbot *et al.*[3] made use of the color reaction produced by pure sulphuric acid to provide a colorimetric method for the quantitative determination of pregnanediol. Guterman[4] modified the Astwood-Jones method to provide for the more rapid qualitative determination of pregnanediol and made it more applicable to clinical use.

The quantitative method described here has the advantage of simplicity thereby making it possible to follow patients over long periods of time by serial determinations permitting the study of the complications of pregnancy and the evaluation of their therapy. The determination of free pregnanediol rather than the conjugated sodium pregnanediol glucuronidate avoids the danger of loss incurred during the urine collection period. Furthermore, other substances than pregnanediol are found in the urine as glucuronidates interfering with the accuracy of the determinations.

During the past year we have carried out over 1500 determinations of pregnanediol in the urine in about 100 patients. Some of these women had normal pregnancies and serial determinations were made throughout their pregnancies and for a week following their deliveries. These women served as normal controls to evaluate the method and to establish basic curves of pregnanediol excretion. The majority of the women had pregnancy complications in whom it was desired to follow the pregnanediol excretion in order to determine any variations from the normal curves in an attempt to establish the role of progesterone metabolism in these complications. In many of these patients daily determinations were possible but in the majority the collections of urine were made 2 and 3 times a week. All of the determinations have been made in duplicate to decrease the likelihood of error in the method.

Method. The women studied for the most part were out-patients who visited this clinic. They were given standard containers for urine collection and were carefully instructed to insure complete 24 hour samples. The first morning specimen on the day of collection was discarded and all urine throughout the day and

* This work has been done under a grant from the Douglas Smith Foundation for Medical Research of the University of Chicago.

1 Venning, E. H., *J. Biol. Chem.*, 1937, **119**, 473.

2 Astwood, E. B., and Jones, G. E. S., *J. Biol. Chem.*, 1941, **137**, 397.

3 Talbot, N. D., Berman, R. A., MacLachlan, E. A., and Wolfe, J. K., *J. Clin. Endocrinol.*, 1941, **1**, 668.

4 Guterman, H. S., *J. Clin. Endocrinol.*, 1945, **5**, 407.

including the first morning specimen the next day were pooled.

The completed collection was brought to the laboratory the same morning and the determination started immediately. All examinations were made the same day they were received. No preservatives were used. In some cases patients were brought into the hospital and urine collections made largely by the patients themselves. It has been our experience that much more accurate 24 hour samples were made by cooperative patients than when collections were left to an already overworked nursing staff. Patients were carefully instructed to report any loss of part of the collections. Samples which for some reason were not complete were discarded. Determinations were made at weekly, biweekly and in some of the more interesting cases, daily intervals.

The chemical procedure employed is essentialy that of Astwood and Jones[2] as modified by Guterman.[4] We have in addition made a few changes of our own for the purpose of increasing the accuracy of the quantitative determination. The technique briefly is as follows:

1. One hundred cubic centimeters of urine, 50 cc of C.P.Toluene, 10 cc of conc. HCl and a few glass beads are added to a 500 cc flat bottomed Florence flask. The flask is connected by a one-holed cork stopper to a vertical reflux condenser and the contents boiled vigorously for 15 minutes on an electric hot plate.

2. The flask it then cooled under tap water to room temperature and its contents transferred to a 500 cc separatory funnel. The lower urine-acid layer is drawn off. The urine-acid layer is shaken twice with 10-15 cc volumes of fresh toluene and returned to the separatory funnel, the urine-acid layer being drawn off between each shaking.

3. The toluene emulsion layer in the separatory funnel is then washed twice with 15 cc portions of 0.1 N NaOH followed by 2 washings with 15 cc portions of distilled H_2O.

4. The washed toluene and toluene water emulsion layers are transferred to a 125 cc Erlenmayer flask. A few glass beads are added. The separatory funnel is rinsed with fresh toluene and the rinsings are added to the flask.

5. The mixture is boiled on an electric hot plate under a hood and when the emulsion layer has disappeared and the toluene is boiling smoothly 10 cc of freshly prepared 2% NaOH in absolute methanol are added slowly. The mixture is boiled until a granular precipitate is obtained and the solution has a yellow or greenish yellow appearance.

6. The toluene mixture is then filtered through fritted glass filters of medium porosity using slight suction. The precipitate is washed with the fresh hot toluene used to rinse out the flask.

7. The combined filtrates are evaporated in a hood utilizing a hot plate. The last traces of toluene are eliminated by means of an air stream. This avoids charring the residue.

8. Five cc of acetone are then added to the residue and the measure gently heated until solution is complete. Twenty cubic centimeters of 0.1 N NaOH are slowly added while the flask is still on the hot plate. When boiling occurs the flask is placed in the refrigerator overnight.

9. The precipitate that forms is collected by filtering through a fritted glass filter and washed with the rinsings of the flask using distilled H_2O. The precipitate is then washed with 10 cc of petroleum ether.

10. The receiving flask is changed and the precipitate dissolved by passing 10 cc of hot absolute ethanol through the funnel. If the precipitate shows any discoloration of a reddish or yellowish tint it is reprecipitated by adding 40 cc of distilled H_2O to the alcohol solution and heated to boiling. This last step is repeated until the precipitate is white.

11. The alcoholic filtrate is evaporated to dryness on a hot plate utilizing an air jet to remove the last few cubic centimeters.

12. Ten cc of C.P. H_2SO_4 are added to the dried white precipitate and allowed to stand one hour for full color development. After proper dilution (usually 1-10) the solution is read in a Coleman model 11 Spectrophotometer at a wave length of 420μ. The readings are interpolated on a curve made by using pure pregnanediol.

In order to test the accuracy of our quan-

TABLE I.
Comparison of Venning Gravimetric Method and the Modified Guterman Method.

No.	Pregnanediol	NaPG as Pregnanediol
1	78.1	78.3
2	68.4	60.1
3	65.7	67.9
4	18.2	16.3
5	9.1	8.1

titative results a few determinations using our technique and the original Venning method were run on the same samples. Fig. 1 shows the close agreement between the two methods.
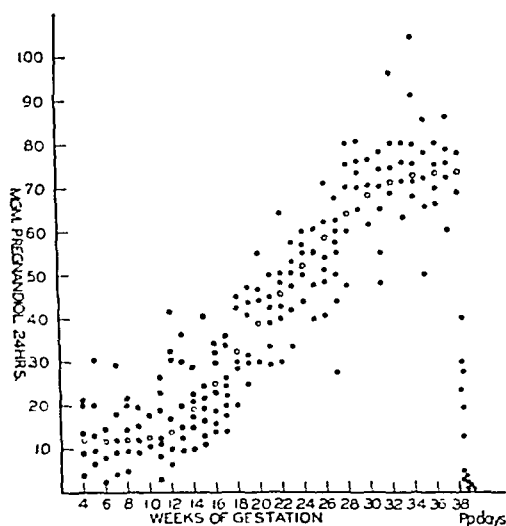
FIG. 1.

A composite curve demonstrating the excretion of pregnandiol in a group of patients during normal pregnancy and the immediate postpartum period.

Normal Pregnancy Curve. There is considerable variation in the daily output of pregnanediol in the individual with a normal pregnancy but the curve follows a typical pattern. During the first 12 to 14 weeks of the gestation the daily excretion will vary from a low of 5 or 6 mg to a high of 20 mg. Some individuals excrete more pregnanediol throughout the pregnancy than do others. The daily output increases slowly during the second trimester of pregnancy leveling off at about the twenty-eighth week of the gestation. The level remains fairly constant during the last 10 weeks of the pregnancy, varying from 70 to 90 mg per day. Individual daily amounts

may fluctuate rather widely reaching as high as 120 mg or as low as 50 or 60 mg. Serial determinations on the same patient are extremely important for only in this way can one recognize sudden changes in the normal pattern. No sudden drop in the level of free pregnanediol prior to the onset of labor has been recognized in our curves although there may be some recognizable decrease in the daily output during the last few weeks prior to the onset of labor.

Following delivery there is a sudden drop in the excretion of pregnanediol. The first 24 hour urine collection may contain as little as half the amount present on the previous day. No more than 10 to 20 mg are eliminated during the second 24 hours. Small amounts varying from 2 to 3 mg may be present for an additional day or two, following which little is excreted. We have collected all urine in our postpartum patients by means of an inlying catheter to provide for complete samples. (Fig. 1)

Most authors agree that pregnanediol is the urinary metabolite of progesterone. During the latter half of the menstrual cycle and the early part of pregnancy, the corpus luteum is the principal source of this hormone. However, with the development of the placenta this organ becomes the principal source of the progestational hormone. It may serve as the only source for in 2 instances in our series as well as in the experience of others (Seegar and Delfs)[5] the removal of the ovary containing the corpus luteum did not alter appreciably the excretion of pregnanediol.

Placental functions must be directly related to the quantitative production of progesterone and the output of the biologically inert steroid pregnanediol. In that the placenta is primarily a circulatory organ providing the circulation link between mother and fetus, the excretion of pregnanediol depends indirectly on the efficiency of placental circulation. Complications of pregnancy associated with disturbances of placental function must invariably be reflected in disturbed pregnanediol excretion. It is not surprising that decreased amounts have been reported in patients who

5 Seegar, G. E., and Delfs, E., *J. A. M. A.*, 1940, 115, 1267.

threaten to abort, in the toxemias of pregnancy, in premature labor, in late death of the fetus. Before intelligent therapy of these complications can be developed, the relationship of progesterone metabolism to these conditions must be understood.

Summary.—A rapid, accurate colorimetric method for the quantitative determination of pregnanediol based on the methods of Venning, Talbot and Guterman is described. Serial determinations in normal pregnancy and pregnancy complications have been carried out in over 100 patients. Pregnanediol excretion in the last 28 weeks of the gestation can be used as a quantitative measure of uteroplacental circulation. During this period the placenta is the chief source of this urinary metabolite for corpus luteum activity wanes rapidly after the first trimester. Serial determinations in normal pregnancy and the complications of pregnancy may throw light on the adequacy of the placenta as the essential organ for the survival of the fetus.

15976

Symmetrical Patterns of Bacteriophage Production.

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In a previous note[1] it was shown that the problem of how bacteriophages are produced can be directly approached through the electron microscopy of metal shadowed "replicas" of the surfaces of agar cultures on which bacteria and bacteriophage are growing together. Such studies of several different bacteriophages are showing many of the phenomena involved in the production of these virus-like objects. The processes are complex and depend not only on the type of bacterium and the strain of bacteriophage but also on such factors as the rate and the duration of growth of the culture. Detailed evidence furnished by the electron microscope will be described elsewhere.

One of the most impressive aspects of the development of bacteriophage from bacterial protoplasm is its completeness, and in certain instances its regularities. With the T3 bacteriophage against the colon bacillus, the pattern of this conversion shows a symmetry as perfect as that of the molecular particles in a crystalline array[2] (Fig. 1). As in this figure the pattern often covers the entire surface of a bacterium; but it is also to be seen spreading throughout extruded protoplasm (Fig. 2). It extends into and includes the thick masses which were bipolar bodies in the original cells. When most clearly visible, the pattern is one of concavities but in many places the separate indentations are filled with spherical bodies having the size of free bacteriophage particles. Photographs have been obtained of this honeycomb structure starting to form within cells that otherwise seem normal; but it is often difficult to be sure whether these indentations are places where fully formed particles have escaped or where relatively immature particles are just beginning to form.

Correspondingly complete conversion to bacteriophage has been observed with other strains but highly symmetrical nets of particles have not been seen following infection with any of the "tailed" bacteriophages.

The many electron micrographs already made suggest far-reaching speculations into the nature of bacteriophages and of any viruses that may exhibit similar mechanisms of increase; but the techniques being used offer opportunities for gaining further information that make discussions of this nature seem premature.

1 Edwards, O. F., and Wyckoff, R. W. G., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 16.

2 Price, W. C., and Wyckoff, R. W. G., *Nature*, 1946, **157**, 764; Markham, R., Smith, K. M., and Wyckoff, R. W. G., *ibid.*, 1947, **159**, in press.

FIG. 1.

A chromium-shadowed electron micrograph of a colon bacillus infected with the T3 strain of bacteriophage. The regular pattern of concavities extends over the entire surface of the organism. Magnification *ca.* 40,000.
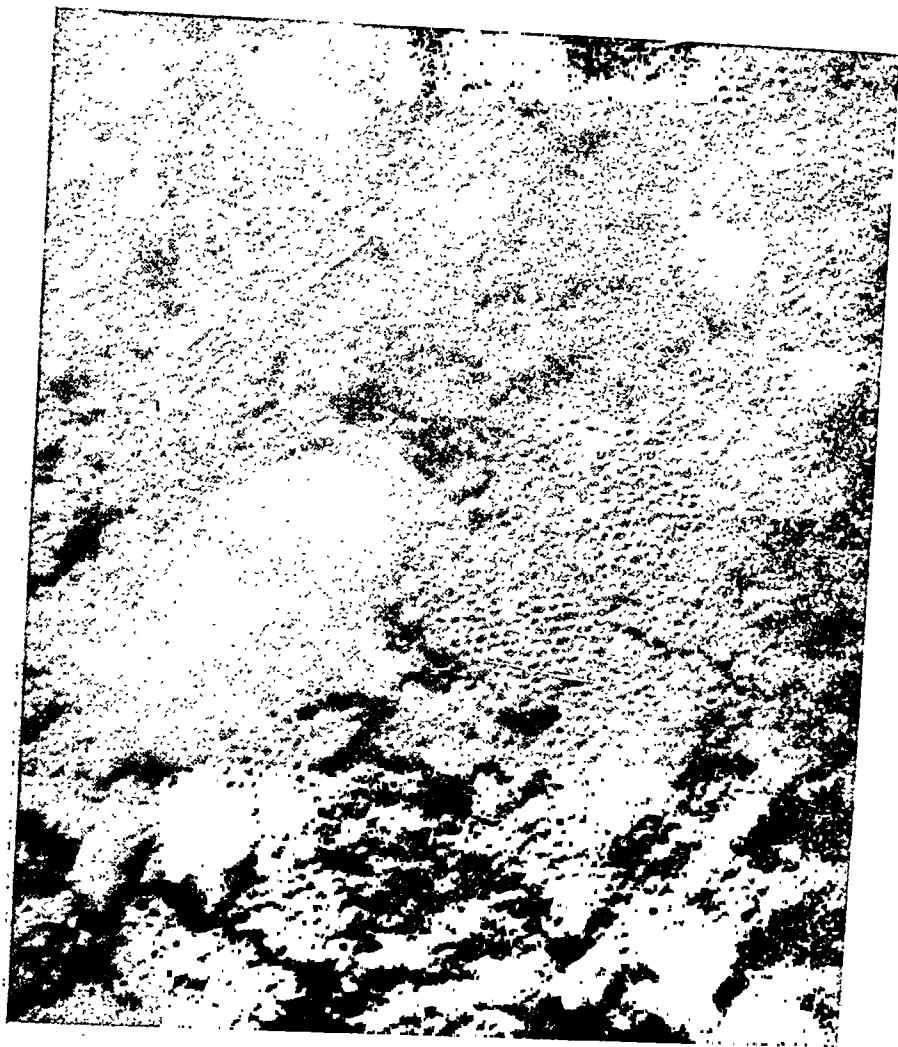
FIG. 2.

A region of bacterial protoplasm left by the lysis of colon bacilli infected with the T3 strain of bacteriophage. An intact bacterium lies to the left of center of the field. Magnification *ca.* 30,000.

15977 P

Gaseous Distention in the Obstructed Small Intestine of Cats.

Fritz Schweinburg, Edward Frank, Arnold Segel, and Jacob Fine.

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In a previous paper[1] we demonstrated that sulfathalidine and sulfamethazine exert an inhibiting effect on gas production in the obstructed small intestine of the cat. This report concerns similar studies of other sulfonamides and of penicillin and streptomycin.

The following drugs were investigated: Sulfadiazine, sulfamerazine, sulfathiazole, sulfasuccidine, penicillin and streptomycin. Phthalic acid and succinic acid were tested because they are hydrolytic products of sulfathalidine and sulfasuccidine.[2] Additional experiments with sulfathalidine and sulfamethazine in smaller doses were carried out and are compared with the previously reported results with the larger doses.

Method. The experimental method is identical with that used previously. Cats weighing 1½-3 kg, which had been deprived of food and water for 24 hours, were subjected to laparotomy with the usual sterile precautions. A heavy cotton ligature was used to completely occlude the esophagus at the cardio-esophageal junction. A similar ligature was applied to the ileum just proximal to the ileocecal junction, thus producing a closed loop. Although the stomach was free of solids or liquids, gas up to 30 cc was present occasionally. This was aspirated prior to injection of the material to be studied. Two teaspoonfuls of powdered malted milk* in 100 cc of milk, with or without the drug to be tested, was injected into the stomach. The dose of sulfonamide was 1.5 g/kilo in most experiments; in the remaining ones, 0.5 g/kilo. The dose of penicillin was 30,000 U/kilo and of streptomycin 60,000 U/kilo. After 22-26

hours the animals were sacrificed by etherization and the amount of gas and liquid or semisolid contained in the closed loop was measured.

A total of 178 cats were studied. Six cats, not included in the results, were found dead after 24 hours. Most of these had received no drug. They showed enormous gaseous distention as the probable cause of death.

Results. Table I lists the average amounts of gas found and also the greatest and smallest amounts, to demonstrate the range of effectiveness of the various drugs used. No relation whatever was apparent between the amount of gas and the amount of solid and liquid material.

The data show that sulfadiazine, sulfamerazine, sulfamethazine, sulfathiazole and sulfathalidine are equally efficacious in depressing gas formation from malted milk in a closed loop. Doses of 0.5 g/kilo are just as effective as the larger dose. Both doses are large compared to amounts used clinically. Sulfasuccidine is definitely less effective, though significantly better than the controls. Succinic and phthalic acid have no effect whatever on gas production. Of the drugs studied, penicillin proved to be the most powerful inhibitor of gas formation. Streptomycin, on the whole, showed a good depressing effect, but the amounts of gas varied over a wide range.

Discussion. Since the sulfonamides in general exert their action on the gram negative bacteria of the intestinal tract (coli-aerogenes group) and on the clostridia normally present there, it is not surprising that these drugs work very much alike in depressing gas formation.

Of the 2 conjugated sulfonamides studied sulfathalidine exerts a definite antibacterial action *in vitro* and in the gut[2,3] while sulfa-

[1] Segel, A., Schweinburg, F., and Fine, J., Proc. Soc. Exp. Biol. and Med., 1946, 63, 17.

[2] Poth, E. J., and Ross, C. A., *Texas Reports on Biol. and Med.*, 1943, 1, 345.

* Wander Powdered Malted Milk.

TABLE I.

Drug	Dose, g/k	No. of cats	cc gas		
			Max.	Min.	Avg
Sulfadiazine	1.5	10	35	4	14.3
,,	.5	10	32	6	16.9
Sulfamerazine	1.5	10	30	1	12.8
Sulfamethazine	1.5	10	30	0	12.0
,,	.5	4	14	8	10.0
Sulfathiazole	1.5	6	22	7	12.0
,,	.5	12	25	1	11.1
Sulfathalidine	1.5	20	60	0	17.5
,,	.5	4	31	5	15.2
Phthalic acid	.6	5	210	87	155.0
Sulfasuccidine	1.5	12	175	13	44.0
Succinic acid	.5	5	164	62	101
Penicillin	30.0 U/kg	10	38	0	8.5
Streptomycin	60.0 U/kg	29	225	0	33
Controls	—	31	215	55	117

succidine does not act *in vitro* but acts in the gut only after hydrolysis.[4] This fact explains the less effective action of sulfasuccidine. Phthalic and succinic acid exert no inhibitory action.

Since penicillin has no effect, except in excessive doses, on gram negative bacilli, it acts presumably by suppressing the clostridia which are present in the gut.

Since coliform bacteria predominate and clostridia are relatively few, one would expect gas formation to be suppressed to a lesser degree by penicillin than by those drugs which work on both clostridia and gram negative bacilli. This is not the case, however, since it works best of all the drugs studied.

The results with streptomycin are difficult to explain. The range of gas production is fairly narrow for all drugs except streptomycin. Of 29 cats treated with streptomycin, 16 had either no gas or less than 5 cc; 6 had less than 25 cc; 3 showed amounts of gas between 25 and 100 cc and 4 had 110, 150, 195, and 225 cc of gas respectively. The last of these is a little more than the highest amount of gas found in the controls, or with phthalic and succinic acid, which may also be considered controls. We are not able to explain these observations.

A study of the chemical composition of the gas and the associated changes in the bacterial flora of the small intestine of dogs brought about by these drugs is proceeding.

Conclusions. Gas formation from malted milk in milk in a closed loop, comprising the stomach and the entire small intestine, is markedly depressed by sulfadiazine, sulfamerazine, sulfamethazine, sulfathiazole and sulfathalidine. There is a definitely less, but still significant depression by sulfasuccidine. Phthalic and succinic acid have no inhibitory effect on gas production. Streptomycin works well in the majority of the experiments, but is a complete failure in others. Penicillin works best of all the drugs tested.

[3] Schweinburg, F. B., and Yetwin, I. J., *J. Bact.*, 1945, **49**, 193.

[4] Poth, E. J., and Knotts, F. L., *Arch. Surg.*, 1942, **44**, 208.

15978

Urinary Excretion of Radioactive Iodine, I131, in a Case of Severe Hyperthyroidism.*

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Earlier studies of Hamilton,[1] Hertz and Roberts,[2] Chapman and Evans[3] and others have provided helpful data with respect to the absorption of I130, its deposition in the thyroid, elimination, and effectiveness in controlling the symptoms in patients suffering with hyperthyroidism. This isotope, obtained from the cyclotron, has a half-life of 12 hours. The isotope, I131, which can be obtained carrier-free from the uranium pile has a half-life of 8 days. The irradiation is, therefore, distributed over a much longer time when I131 is used for therapeutic purposes. The amount of I131 required for treatment needs to be determined and little data is as yet available in the literature with respect to its use in the treatment of hyperthyroidism. The purpose of the present report is to provide data concerning the excretion and retention of I131.

Radioactive iodine,† I131, was administered orally to a female patient, age 29, suffering from a severe hyperthyroidism of diffuse type with marked exophthalmos. Lugol's solution had been given for a period of several months but had produced only brief improvement and was discontinued 3 weeks before the first dose of radioactive iodine was administered. The patient proved to be sensitive to thiouracil and she was not in good enough condition to tolerate surgery. Her weight had gone down to 87 pounds and the BMR was +70.

The weight of the thyroid was estimated to be 50 g.

A dose of 9.1 millicuries of I131 was given on October 11, 1946. No toxic reaction was observed. Urinary excretion was determined by means of a beta-ray Geiger-Muller Counter.[4] In the first four 12-hour intervals it was respectively 10.4%, 15.5%, 10.4%, and 8.5% (in per cent of I131 given), thus indicating a total retention after 2 days of 54%. A dose of 3 millicuries was given on November 24, 1946. Urinary excretion in the first two 12-hour intervals was respectively (in per cent of I131 given) 12.7% and 1.8% giving a total retention after 1 day of 85.5%.

Calculated values of retention of iodine in the body are given in Table I, and measurements on excretion of I131 in the urine are given in Fig. 1. Iodine retention is given in per cent of the original dose. (Both radioactive decay and excretion factors being considered.) Excretion is plotted in per cent of the iodine retained at a time half-way between the collection of specimens.

The question has been raised of the advisability of giving stable iodine following the administration of the radioactive isotope. For this reason, stable iodine was administered as Lugol's solution for a period of 2 days after the urinary excretion of I131 had reached a low level. (The continued low level of excretion of I131 has been explained on the basis of decay of thyroxin in the blood.) Administration of Lugol's solution was followed, each time, by excretion of I131 in relatively large amounts. The excretion of radioactive iodine seemed to begin some 24 hours after the stable iodine was given. This delay is quite different from the immediate excretion (in the first 12-hour

1 Hamilton, J. G., *Radiology*, 1942, 39, 641.

2 Hertz, S., and Roberts, A., *J. Am. Med. Assn.*, 1946, 131, 81.

3 Chapman, E. M., and Evans, R. D., *J. Am. Med. Assn.*, 1946, 131, 86.

† The radioactive iodine was obtained through the cooperation of the Isotope Branch, Research Division, United States Engineering Office, Manhattan District, Oak Ridge, Tenn.

4 Wang, J. C., Marvin, J. F., and Stenstrom, K. W., *Rev. Scient. Inst.*, 1942, 13, 81.

TABLE I.

| Interval, days | % of administered I[131] retained (at midpoint of interval) | | Equivalent roentgens per interval | |
	I—9.15 mc (100%) (.18 mc/g thyroid)	II—3.0 mc (100%) (.06 mc/g thyroid)	I	II
0 -0.5	93.5	91.5	1040	330
0.5-1.0	78	82	870	300
1.0-1.5	61	76	680	275
1.5-2.5	51	70	1140	510
2.5-3.5	43	64	960	470
3.5-4.5	38	58	850	425
4.5-5.5	34	51	760	370
5.5-6.5	30	43	670	315
6.5-7.5	24	37	535	270
7.5-8.5	17.5	33	390	240
8.5-9.5	13	30	290	220
Estimated additional dosage (extended over a period of several weeks)			1765	1775
Total dose in equivalent roentgens			9950	5500

interval) following administration of radio-active iodine. The same experiment was carried out in one case of carcinoma of the thyroid with metastasis (unpublished).)In this case, however, administration of Lugol's solution did not result in increased excretion of I[131].

The estimated dose in roentgens for each 12-hr or 24-hr period is given in Table I. This data was calculated in accordance with
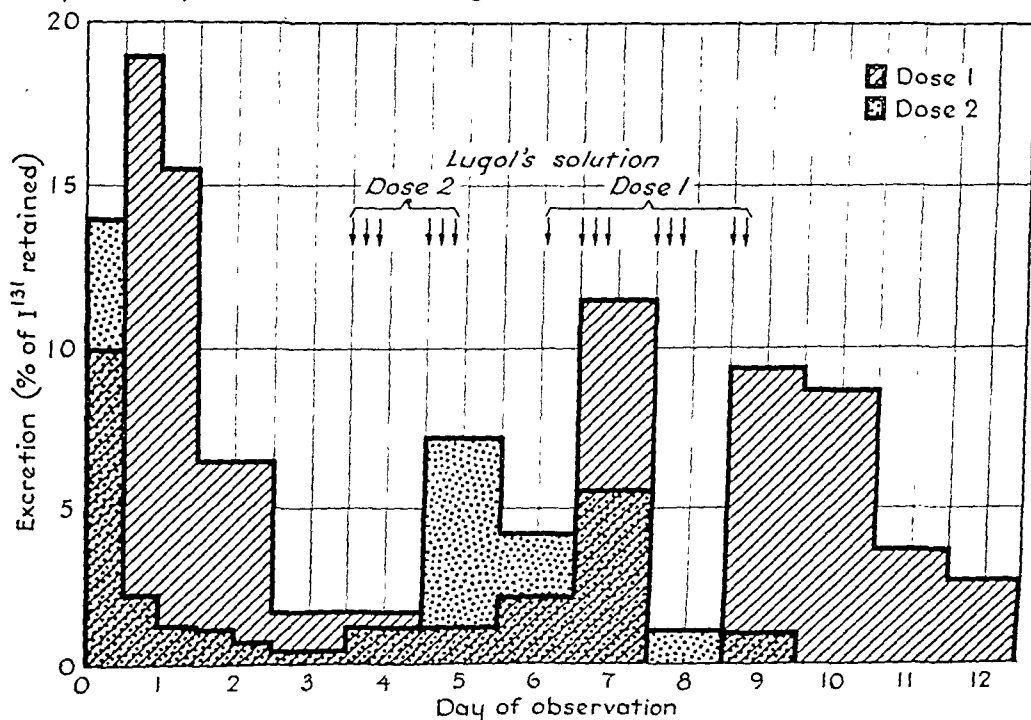
FIG. 1.
Urinary excretion of I[131] per 12 hours calculated in per cent of iodine retained at a time half-way between the collection of specimens. Dose 1 was 9 mc. Dose 2 was 3 mc, one month later. No data were available for the 8th day following dose 1 as the specimen was lost. Arrows indicate the time of administration of Lugol's solution.

Evans[3] formula, on the basis of a thyroid weight of 50 g. (The table gives estimates of the dose per 12 or 24-hour interval and of the total dose). The total dose is estimated to be 9950 roentgens for the first administration of 9.1 millicuries, and 5500 roentgens for the second administration of 3 millicuries. It should be noted that the total dose in roentgens following the second administration (3 millicuries) agrees with the simplified formula of Hertz and Roberts,[2]

roentgens eqivalent

$$= 117{,}000 \times \frac{\text{millicuries } I^{131}}{\text{grams thyroid}}$$

It is too early to evaluate the result of the treatment. Though the patient has improved somewhat and has gained weight, no decided reduction of the BMR occurred in the first 2 months following the first dose, and it seems probable that further treatment will be required.

Summary. Information is given of the urinary excretion of radioactive iodine following administration of relatively large doses in a patient with severe hyperthyroidism. Administration of stable iodine (in Lugol's solution) after the urinary excretion of I^{131} had reached a low level resulted in a pronounced increase in this excretion, which lasted some 48 hours after discontinuing Lugol's solution. This increase in excretion of I^{131} was not observed following administration of Lugol's solution in one case of carcinoma of the thyroid with metastasis. Retention of 85% of the I^{131} by the thyroid agrees with other figures on the retention of I^{130} by the hyperplastic thyroid, when iodine has been withheld for a period of over 4 weeks prior to radioactive iodine.‡

‡ A third dose of 9 millicuries of I^{131} was given the same patient in January, 1947. Loss of iodine in the first 2 days was high (50%). Use of Lugol's solution again resulted in increased excretion of radioactive iodine. The metabolic rate had returned to normal when the patient was next seen in March, 1947.

Two additional cases have since been treated with doses of 9 millicuries of I^{131} (estimated 50 grams thyroid tissue). Excretion was much the same as that following the second dose of iodine for patient No. 1. Use of Lugol's solution resulted in increased excretion of radioactive iodine in both of these cases.

Dr. C. J. Watson, Director of the Department of Internal Medicine, University of Minnesota Hospitals, made the arrangements for the treatment of the patient, and we are obliged to him for the opportunity to carry out these measurements and for valuable advice.

15979 P

The Development of *Fundulus heteroclitus* Embryos in Solutions of Metrazol.

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Speidel[1] has attempted to ascertain the specific effect of metrazol on the nervous system by immersing frog tadpoles in aqueous solutions of it and by studying, by direct microscopic and ciné-photomicrographic methods, the outgrowth of living nerve fibers in the tadpoles' tails. Using a 2% solution for approximately 20 minutes, Speidel found that the growing nerve endings degenerated during the period of immersion, but recovered and subsequently regenerated when the tadpoles were returned to pond-water.

The present communication reports the results of studying the development of cer-

1 Speidel, C. C., *Proc. Am. Philos. Soc.*, 1940, 83, 349.

tain aspects of gross behavior in whole embryos (*Fundulus heteroclitus*) which were immersed in solutions of metrazol at different stages of development and which remained in them for varying periods of time. The investigation was undertaken with the possibility in mind that subjecting embryos to treatment at various stages of development might produce alterations of structure and function in the central nervous system which could be correlated with the particular fiber-tracts being laid down during the period of treatment.

Over 500 eggs of *Fundulus heteroclitus* were treated with aqueous solutions of metrazol (Pentamethylentetrazol, Bilhuber-Knoll Corporation). Solutions, made in distilled water, were used in strengths of either 0.1% or 2%. The embryos were introduced into the solutions at stages varying between 6 and 34 (Oppenheimer[2]), and remained in them for periods varying from a few hours to 14 days. In some cases they remained in the solutions until fixation; in others they were transferred to tap- or sea-water. Some embryos were dechorionated before treatment, others were left in their chorions throughout; still others were dechorionated during the course of treatment or recovery. All embryos were observed at least twice daily; those which survived treatment were preserved and are being prepared for histological study.

The gross effects, which were more drastic in dechorionated embryos than in those with chorions intact, varied also in degree according to the age of the embryos, the strength of the solutions used and the duration of the experiments. In some cases recovery occurred after the embryos were transferred to tap- or sea-water.

None of the embryos treated with 2% metrazol solution before gastrulation developed normally; some of those treated at pregastrular stages with 0.1% solution reached stage 32, the stage of hatching, and completed the major part of their early development. Embryos treated subsequent to gastrulation but before the establishment of circulation developed for as long as 14 days in the 0.1%

solution; of comparable embryos treated with 2% solution, only 2 (both left in their chorions during treatment and transferred from metrazol after 2½ days), reached stage 31.

There was a marked effect on the circulation of embryos treated between stages 23 and 32; the rate of the heartbeat became markedly slowed, and a large vesicle, filled with static blood, formed just distal to the sinus venosus.. In addition, the tonic relationships of the longitudinal body musculature were often affected in embryos treated at these stages. Many of the embryos treated between stages 23 and 26 exhibited sharp localized contractures of the trunk musculature. In addition, many embryos treated between stages 23 and 32, whether or not such localized contractures were present, exhibited various degrees of kyphosis or lordosis which involved sometimes trunk, sometimes tail, sometimes both. Embryos treated between stages 27 and 32 in some cases also showed abnormalities in the action of the mouth and opercular apparatus, which were held open without performing the usual rhythmic movements.

Embryos treated subsequent to stage 32 did not exhibit the same tonic abnormalities which characterized the group just described and were not kyphotic or lordotic. They sometimes, however, in contrast, exhibited sharp lateral contractions and held their tails bent to left or right. Their mouths were almost invariably stretched wide open and exhibited no rhythmic movements. Righting reflexes were lost in these embryos during the period of treatment.

Whether the effect of the drug on the reactions of the embryos acts through the nervous system or directly on the muscular system, and whether or not the drug has resulted in morphological changes within the nervous system that do not produce functional changes in behavior, can be ascertained only after histological examination of the embryos is completed. It is clear, however, that subjecting whole embryos to the action of the drug provides a further method for study of the development of structural and functional relations in the embryonic central nervous system.

[2] Oppenheimer, J. M., *Anat. Rec.*, 1937, *68*, 1.

15980

Effects of Testis Hyaluronidase and Seminal Fluids on the Fertilizing Capacity of Rabbit Spermatozoa.

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An enzyme, extracted from mammalian testis, which increases the permeability of skin and other tissues containing hyaluronic acid is known as hyaluronidase.[1-4] The cumulus cells surrounding the tubal ova of the rabbit were shown to be dispersed in the presence of fairly large numbers of sperms by a heat labile enzyme,[5,6] now identified as hyaluronidase.[7-10] On the basis of these observations, the participation of hyaluronidase in fertilization,[7-10] the capacity of hyaluronidase to increase the fertilizing power of sperms,[11] and the clinical use of hyaluronidase for sterility[12] have been reported. Rowlands' report[11] on increased fertilizing capacity in rabbits was based on the use of heated seminal fluid which contains hyaluronidase. In the following experiments we have examined the effects of purified testis hyaluronidase on fertilization *in vivo*.

Methods. Adult non-pregnant doe rabbits were superovulated according to Pincus.[13]

They were inseminated with a minimal effective number of spermatozoa (number of sperms needed to fertilize only a small number of ova) suspended in different fluids just before the intravenous injection for the induction of ovulation. The rabbits were sacrificed 25 to 30 hours later and the ova were flushed out from the oviducts. The fertilized and unfertilized ova were counted.

The semen of a single male rabbit was collected with an artificial vagina[14] for insemination in order to control the variation of sperm quality between different individuals. The interval between each collection of sperm was 3 to 4 days in order to keep the sperm quality constant. The general method of insemination was carried out according to Walton.[15,16] The semen just after collection was diluted with saline (0.9% NaCl) about 1 part to 1,000. Then the sperm concentration was immediately counted by means of the hemocytometer technique. Saline or sperm was added if the concentration of sperms was too high or too low. Then 0.5 ml of this suspension was added to one of the following: (1) 0.5 ml of saline containing a known amount of hyaluronidase, (2) 0.5 ml of supernatant fluid of normal semen after heating, (3) 0.5 ml of semen from a vasectomized male, (4) 0.5 ml of saline serving as a parallel control. These mixtures (all 1 ml in volume) were introduced into the vagina of each rabbit. Usually, 6 rabbits were inseminated at a time. The time interval between collection of semen and the first insemination was about 10 minutes, and that between the first and last insemination was about 20-30 minutes.

1 McClean, D., *J. Path. and Bact.*, 1930, **33**, 1045.

2 McClean, D., *J. Path. and Bact.*, 1931, **34**, 459.

3 Hoffmann, D. C., and Duran-Reynals, F., *J. Exp. Med.*, 1931, **53**, 387.

4 Chain, E., and Duthie, E. S., *Brit. J. Exp. Path.*, 1940, **21**, 324.

5 Pincus, G., and Enzmann, E. V., *J. Exp. Med.*, 1935, **62**, 665.

6 Pincus, G., and Enzmann, E. V., *J. Exp. Zool.*, 1936, **73**, 195.

7 McClean, D., and Rowlands, I. W., *Nature*, 1942, **150**, 627.

8 Fekete, E., and Duran-Reynals, F., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 119.

9 Leonard, S. L., and Kurzrok, R., *Endocrinology*, 1945, **37**, 171.

10 Swyer, G. I. M., *Lancet*, 1946, **251**, 755.

11 Rowlands, I. W., *Nature*, 1944, **154**, 332.

12 Kurzrok, R., Leonard, S. L., and Conrad, H., *Am. J. Med.*, 1946, **1**, 491.

13 Pincus, G., *Ant. Rec.*, 1940, **77**, 1.

14 Macirone, C., and Walton, A., *J. Agri. Sci.*, 1938, **28**, 122.

15 Walton, A., *Proc. Roy. Soc. B.*, 1927, **101**, 303.

16 Walton, A., *J. Exp. Biol.*, 1930, **7**, 201.

The testis hyaluronidase used was obtained from bull testicles (Schering Corporation). The seminal hyaluronidase was prepared according to Rowlands;[11] i.e., normal semen was heated at 50° C for 8 to 11 minutes to kill sperms, and then kept on ice for 5 minutes and centrifuged. The supernatant fluid was used. After insemination, the heated seminal supernatant fluid and the vasectomized male semen were assayed by the viscosimetric method[17] and expressed as mg of testis hyaluronidase. The number of spermatozoa in the suspension was counted again 4 to 6 times and the average number of sperms inseminated was calculated.

Results. The complete data of these experiments are presented in Table I. It is evident that the fertilizing capacity of spermatozoa was not affected when testis hyaluronidase (1 to 0.65 mg per ml) was added. The average percentage of fertilized ova for 10 experimental does was 30 and that of 10 parallel control does was 27. The total number of fertilized ova in the experimental group was 70 out of 203 (34%), while that in the control group was 48 out of 198 (24%).

The average percentage of fertilized ova (60%) in the 13 experimental does inseminated with supernatant fluid of normal semen which contained hyaluronidase (1.05 to 0.2 mg per ml) was higher than that of 13 parallel control does (38%). But there is no statistical significance of the difference ($t=1.56$, $P<0.2>0.1$). However, the total number of ova fertilized in the experimental group (62%) is higher than that of the parallel control group (39%).

The average percentage of fertilized ova in those 10 does inseminated with sperms suspended in saline and the semen of vasectomized bucks which contains no hyaluronidase was 63, while that of 9 parallel control does was only 15. The difference is statistically significant ($t=4.66$, $P<0.01$). The total number of ova fertilized in the experimental group (65%) is higher as compared with that in the parallel control group (21%).

It is quite clear from these data that the

extra hyaluronidase added to the sperm suspension does not influence the fertilizing capacity of spermatozoa. On the other hand, seminal fluid with or without hyaluronidase does increase the fertilizing capacity of rabbit spermatozoa.

Discussion. It is a common thought that seminal fluid is not important for the fertilizing capacity of spermatozoa because the epididymal spermatozoa,[15,16,18] spermatozoa separated from seminal fluid by centrifugation,[19] and semen in a very diluted form[20,21] are able to insure fertilization. The present investigation, however, reveals clearly that the importance of seminal fluid shows up when the number of spermatozoa is decreased to a minimum. Thus, any disturbances of accessory glands may affect the fertility of a male though clinical data on this point are still scarce.[22]

The great variation in the percentage of fertilized ova per doe (Table I) under strictly controlled experimental conditions leads one to reject those positive conclusions based upon only a few clinical cases or based on some experimental studies without strict control of variations of sperm quality in different individuals and in different time intervals of collection in the study of such an intrinsic process as fertilization.

Although the dispersal of cumulus cells surrounding the ovum by sperms or by hyaluronidase *in vitro* is unquestionable, the role of hyaluronidase in the complicated process of fertilization *in vivo* is still uncertain. Even if hyaluronidase *per se* plays an important role in fertilization, the hyaluronidase of spermatozoa is quite adequate to perform its function without further addition of hyaluronidase.

Summary. Thirty-three doe rabbits were inseminated with a minimal effective number of spermatozoa suspended in saline containing

17 Hadidian, Z., and Pirie, N. W., *Biochem. J.*, in press.

18 Young, W. C., *J. Exp. Biol.*, 1931, **8**, 151.

19 Walton, A., *Proc. Am. Soc. Ani. Prod., 31st Meeting*, 1938, 238.

20 Chang, M. C., *J. Exp. Biol.*, 1946, **22**, 95.

21 Salisbury, G. W., Elliott, I., and Van Demark, N. L., *J. Dairy Sci.*, 1945, **28**, 233.

22 Huggins, C., *The Role of the Accessory Glands in Fertility. Diagnosis in Sterility.* 1946. Edited by E. T. Engle. Charles Thomas, Publisher, Ill.

TABLE I. Effect of Hyaluronidase and of Seminal Fluid on the Fertilizing Capacity of Rabbit Spermatozoa.

Experimental series	Exp. No.	No. of sperms inseminated ×1000	Does No.	Conc. of hyaluronidase mg/ml	Experimental group (E) No. ova				Does No.	Control group (C) No. ova				% difference in fertilization (E-C)
					Fertilized	Unfertilized	Total	% fertilization		Fertilized	Unfertilized	Total	% fertilization	
Testis Hyaluronidase added	2	228	410	1	2	13	15	13	411	18	3	21	86	−17
	3	226	413	"	20	19	39	51	414	2	11	13	15	−15
			416	"	0	28	28	0	417	0	23	23	0	
	4	342	419	"	13	0	13	100	420	7	18	25	28	27
			363		6	31	37	16	365	0	27	27	0	48
	6	50	371	.65	23	6	29	79	378	4	6	10	40	−17
			421		0	13	13	0	423	2	11	13	15	
	8	116	424	"	0	11	11	0	426	1	29	30	3	
			337		6	10	16	38	434	7	13	20	35	3
			436		0	2	2	0	437	7	9	16	44	
Avg % of fertilization per doe / Total No. of ova					70	133	203	34		48	150	198	24	10
Supernatant fluid of heated normal semen added	5	274	394	.625	20	0	20	100	393	23	0	23	100	2
			397		31	3	34	91	400	12	1	13	92	1
	6	50	422	.20	0	18	18	0	423	2	11	13	15	
			425		3	16	19	16	426	1	29	30	3	3
	7	111	472	.325	19	11	30	63	428	14	2	16	88	
			431		5	5	10	50	432	0	6	6	0	50
	8	116	433	.75	12	0	12	100	434	7	13	20	35	34
			435		5	2	7	71	437	7	9	16	44	
	9	96	444	.56	10	0	10	100	445	2	12	14	14	59
			446		1	12	13	8	447	1	7	8	13	3
	10	130	456	.5	16	7	23	70	458	3	24	27	11	
	11	173	460	1.05	10	6	16	63	461	1	18	19	5	22
			463		1	1	2	50	464	18	8	26	69	23
Avg % of fertilization per doe / Total No. of ova					133	81	214	62		91	140	231	39	23
Semen from vasectomized bucks added	10	130	454	.051	16	0	16	100	455	0	11	11	0	78
			457		22	6	28	79	458	3	24	27	11	
	11	173	459	0	11	8	19	58	461	1	18	19	5	16
			462		7	5	12	58	464	18	8	26	69	
	12	150	467	0	4	4	8	50	466	1	12	13	8	49
			469		24	8	32	75	468	1	26	27	4	
	17	125	508	0	19	8	27	70	470	13	19	32	41	
			511		6	17	23	26	510	0	16	16	0	53
			509*		10	3	13	77	513	0	9	9	0	
			512*		6	9	15	40						
Avg % of fertilization per doe / Total No. of ova					125	68	193	65		37	143	180	21	44

*Semen of vasectomized male heated at 50°C for 20 minutes was used. † Very few dead sperms present.

purified testis hyaluronidase, or saline and supernatant fluid of heated normal semen containing hyaluronidase, or in saline and semen of vasectomized buck containing no hyaluronidase. *Thirty-two does were inseminated at the same time with the same number of sperms collected from the same rabbit but suspended in saline, serving as parallel controls. It was found that it was the seminal fluid, not* hyaluronidase, which really increased the fertilizing capacity of spermatozoa.

15981

Range of Antibiotic Activity of Protoanemonin.*†

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Extracts of buttercups and *Anemone pulsatilla* exert an inhibitory action on the growth of a number of pathogenic bacteria.[1,2] The active principle extracted from *A. pulsatilla* proved to be protoanemonin.[3] The present studies deal with the titration of the antibiotic activity of protoanemonin when tested against a variety of bacteria and fungi, as well as a few representatives among the viruses and protozoa.

Methods of testing the bacteria. The protoanemonin used in these experiments was extracted from dried ground *A. pulsatilla* or was

1 Seegal, B. C., and Holden, M., *Science*, 1945, **101**, 413.

2 Carlson, H. J., Bissell, H. D., and Mueller, M. G., *J. Bact.*, 1946, **52**, 155.

3 Baer, H., Holden, M., and Seegal, B. C., *J. Biol. Chem.*, 1946, **162**, 65.

prepared synthetically, as described previously.[3] The stock was a 1-100 dilution by volume in sterile distilled water from which further dilutions in the test media were prepared.

The *in vitro* susceptibility of the bacteria, with the exception of the Mycobacteria, was determined by inoculating 0.5 cc of a 10^{-3} *dilution of a 24- or 48-hour culture of the test organisms into 4.5 cc of media containing decreasing concentrations of the antibiotic. The media chosen, as indicated in the tables, were those which provided favorable growth conditions for the bacteria under investigation. In some cases the organisms were tested when grown in each of 2 media. After a period of incubation at 37°C, sufficient for optimum growth of the control tubes, containing no protoanemonin, the cultures were examined for the presence of visible turbidity or other evidence of growth, such as the production of a pellicle, pigment or gas. The maximum dilution of protoanemonin capable of preventing the appearance of growth in the period of time specified is recorded in the* tables.

Testing of the Mycobacteria required modifications in technique. *A loopful of pellicle 1 cm in diameter from a 25-day-old culture was used to inoculate 100 cc volumes of*

TABLE I.

The Maximum Dilution of Protoanemonin Preventing the Visible Growth of a Number of Gram-positive Aerobic and Anaerobic Bacteria.*

Organism	Dilution of protoanemonin × 1000	Media used	Time of incubation
Strep. hem. Group A (3 strains)	52-66	1% chicken serum meat infusion broth	Overnight (approx. 16 hr)
Strep. hem. Group D (2 strains)	16-20	,, ,, ,, ,, ,, ,,	,,
Strep. viridans (2 strains)	33-55	,, ,, ,, ,, ,, ,,	,,
D. pneumoniæ Types I, II, III, VII	55	,, ,, ,, ,, ,, ,,	,,
Staph. Oxford H	60-83	Meat infusion broth	,,
Staph. Oxford H	100-150	Casein hydrolysate broth	,,
Staph. albus	66	Meat infusion broth	,,
M. lysodeikticus	44	,, ,, ,,	,,
C. diphtheriæ	75-100	0.5% glucose meat infusion broth	,,
C. hoffmanni	75-100	,, ,, ,, ,, ,,	,,
C. xerosis	6-12	,, ,, ,, ,, ,,	,,
C. xerosis	16-30	Casein hydrolysate broth	,,
My. tuberculosis hominis	166	Sauton's	1 month
My. tuberculosis hominis	100-330	Dubos'	4 days
My. tuberculosis bovis	100-250	,,	,,
My. tuberculosis avium	250-450	,,	,,
B. subtilis (contains spores)	20-50	Meat infusion broth	Overnight (approx. 16 hr)
B. anthracis (contains spores)	20-50	,, ,, ,,	,,
Cl. histolyticus	30-350	0.1% agar in 1% glucose meat infusion broth	48 hours
Cl. tetani	100-120	,, ,, ,,	,,
Cl. novyi	30-60	,, ,, ,,	,,
Cl. welchii	30-350	,, ,, ,,	,,
Cl. oedematiens	50-100	,, ,, ,,	,,
Cl. sporogenes	30-350	,, ,, ,,	,,

* All organisms were tested repeatedly. When results varied from day to day the extremes of variation are given.

Sauton's media containing the appropriate dilutions of protoanemonin. When Dubos'[4] medium was used 0.1 cc of a 5-day-old culture was the inoculum employed to seed the 5 cc of test medium.

All tests were repeated at least once and usually several times. If the concentration of protoanemonin causing inhibition of growth varied on different days the range of activity is indicated in the tables.

Results with bacteria. In Tables I and II it may be seen that all the bacteria tested possessed some degree of sensitivity to the antibiotic action of protoanemonin. However, the maximum inhibiting dilution varied widely. One strain of *Corynebacterium xerosis* required a 1-6000 dilution to prevent growth, while the Mycobacteria were inhibited by approximately one-thirtieth of this amount of protoanemonin. It is interesting also that

many of the Gram-positive bacteria were somewhat less sensitive that the Gram-negative organisms. Those organisms tested in both broth and casein hydrolysate medium[3] showed greated sensitivity to protoanemonin when grown in the semi-synthetic medium.

Experiments were undertaken to determine whether the acidity of the medium, the size of the inoculum and the age of the culture might be contributing factors in the susceptibility of bacteria to protoanemonin. First, the possibility that the acidity of the medium might influence the inhibiting action of protoanemonin was investigated, using *Escherichia coli* as the test organism and hydrogen ion values ranging from pH 6.7 to pH 7.8. In neither meat infusion broth nor casein hydrolysate broth, with or without the addition of 1% glucose, was there a change in the end titer of the protoanemonin.

The size of the inoculum was investigated, using 4 organisms, *Staphylococcus oxford* H, *Corynebacterium xerosis, Escherichia coli* and

4 Dubos, R. J., and Davis, B. D., *J. Exp. Med.*, 1946, **83**, 409.

TABLE II.

The Maximum Dilution of Protoanemonin Preventing the Visible Growth of a Number of Gram-negative Aerobic Bacteria. All Tests Were Incubated Overnight (Approximately 16 Hours).*

Organism	Dilution of protoanemonin × 1000	Media used
K. pneumoniæ	40	1% chicken serum meat infusion broth
N. catarrhalis	166	Glucose broth
Ps. aerugenosa (3 strains)	30-100	Meat infusion broth
,, ,, ,, ,,	40-200	Casein hydrolysate broth
Scr. marcescens	80-180	Meat infusion broth
V. choleræ	60-100	,, ,, ,,
,, ,,	200	Casein hydrolysate broth
P. vulgaris	80	Meat infusion broth
P. OX19	120	,, ,, ,,
Alk. fecalis (2 strains)	60-80	,, ,, ,,
Es. communis	50	,, ,, ,,
Es. communior	50	,, ,, ,,
,, ,,	100	Casein hydrolysate broth
Eb. typhi "O" and "H"	250-330	Meat infusion broth
S. paratyphii	166	,, ,, ,,
S. Schottmülleri	166	,, ,, ,,
Sh. dysenteriæ Shiga	250	,, ,, ,,
,, ,, Flexner	166	,, ,, ,,
,, ,, Sonne	166	,, ,, ,,

* See footnote Table I.

Vibrio cholerae. Sixteen-hour cultures, undiluted and diluted 10^{-1}, 10^{-3} and 10^{-7}, were added to casein hydrolysate broth and to meat infusion broth, both media containing varying amounts of the antibiotic. The findings indicated that the antibacterial activity was independent of the size of the inoculum when the inoculum was diluted 10^{-1}, 10^{-3} or 10^{-7}, however the undiluted inoculum was not consistently inhibited by similar dilutions of protoanemonin.

The effect of the age of the inocula upon the susceptibility of the same 4 organisms was determined by using 2-, 4-, 6-, 16-, and 48-hour cultures. Each culture was diluted until the turbidity approximated that of the 2-hour culture, and 0.5 cc served as the inoculum. The number of viable organisms was determined by pouring plates from each tube and counting colonies. The variation in number of viable organisms was well within the limits of what was found to be without effect on the outcome of the test. The results showed that the end titer of protoanemonin was the same irrespective of the age of the inoculum used.

Methods and results with fungi. Three yeasts, a non-pathogenic *Saccharomyces cerevisiae* and the pathogenic *Candida albicans* and *Cryptococcus neoformans,* were grown in 1% glucose broth for 2 to 5 days. Five-tenths cubic centimeters of a 10^{-3} dilution of the culture was added to 4.5 cc of the glucose broth containing varying amounts of the antibiotic. The dermatophytes or ringworm fungi were cultivated in honey broth for 10 days until a luxuriant mycelium was formed. The mycelium and spores were triturated in a mortar with sterile saline and one drop of the suspension added to the glucose broth tubes. In the case of *Coccidioides immitis,* broth was added to cover a honey agar slant culture and, after repeated pipettings to free the culture, 2 drops of the broth were added to each of the tubes of glucose broth containing the dilutions of protoanemonin. The last 3 fungi tested— *Allescheria boydii (Monosporium apiospermum),* an unidentified mold from a box of strawberries, and another from an old orange —were inoculated into glucose broth by simply touching the mycelial growth with a platinum loop and transferring it to the test media. When the growth in the control tubes was abundant, the amount of protoanemonin required to give inhibition of growth of the fungi was noted. The time of incubation varied with the different species (Table III).

These experiments show that protoanemonin inhibits the growth of fungi, which proved to be as sensitive as the bacteria. The inhibit-

TABLE III.
Maximum Dilution of Protoanemonin Preventing the Visible Growth of a Number of Fungi Grown in Glucose Broth.*

Organism	Dilution of protoanemonin, × 1000	Time of reading, days
Saccharomyces cerevisiæ	50-166	2
Candida albicans (2 strains)	100-200	2
Cryptococcus neoformans (4 strains)	83-300	2
Trichophyton mentagrophyles (gypseum)	125	5
Microsporium canis	125	5
Microsporium audouini	62-83	5
Trichophyton purpureum	83	5
Coccidioides immitis	125	2 ?
Alleschcria boydii	166	5
Unidentified mold from strawberry plant	62	1
Unidentified mold from orange	62	2

* See footnote Table I.

ing concentration of protoanemonin varied from 1-50,000, in the case of *Saccharomyces cerevisiae*, to 1-300,000 for *Cryptococcus neoformans*.

Methods and results with protozoa. Tetrahymena geleii[5] and *Trypanosome gambiense* were two protozoa tested for their sensitivity to protoanemonin. Four-day cultures of *Tetrahymena*, grown in 2% bactopeptone or proteose peptone at room temperature (26°-28°C), were added in 0.5 cc amounts of 4.5 cc of peptone broth containing dilutions of protoanemonin varying from 1-200,000 to 1-500,000. In the tube containing 1-200,000 dilution of protoanemonin only a very occasional sluggish, round, small organism with many fine granules might be seen after one day, while in 2 days none were discernible. The 1-300,000 dilution produced a markedly modified growth but did not usually kill. At the end of 2 days the organisms were still small and far less active. Morphologically the stoma was hard to distinguish and the granules appeared smaller and more numerous than in the control organisms. The protozoa in the tubes containing protoanemonin diluted 1-400,000 and 1-500,000 showed increasingly less variation from the normal growth, which was distinguished by the appearance of many actively mobile organisms with several undergoing fission in each field.

The sensitivity of *Trypanosome gambiense* was investigated by inoculating 2 drops of citrated infected guinea pig blood into 1 or 2 cc amounts of Weinman's semi-solid cell free medium[6] containing different concentrations of protoanemonin. The cultures were incubated at room temperature (26°-28°C). The trypanosomes in the control tubes had grown in 5 days after inoculation. No trypanosomes were demonstrable in dilutions of protoanemonin 1-200,000 at the end of 5 days, whereas active organisms were present in the 1-400,000 dilutions which appeared similar to those organisms in the control tubes (Table IV).

In other tests the inoculum was a drop from a 9-day culture of *T. gambiense*. Growth of trypanosomes was absent in the 1-1,600,000 dilution after 5 days of observation.

Methods and results with bacteriophages. An investigation was undertaken to determine the effects of protoanemonin on coli and on staphylococcus bacteriophage. Two sets of tubes containing protoanemonin diluted 1-2000 in bacteriophage were prepared. One set was incubated at 37° C for 1½ hours, the other set remained at room temperature over night. Serial dilutions in broth from 10^{-1} to 10^{-9} were then seeded with their respective organisms. In the controls distilled water was substituted for protoanemonin. The bacteriophage titer was the same in all the tests, the ones in which the bacteriophages were first incubated with the antibiotic and those in which distilled water was substituted for pro-

5 Furgason, W. H., *Arch. Protistenkunde*, 1940, 94, 224.

6 Weinman, D., Proc. Soc. Exp. Biol. and Med., 1944, 55, 82.

TABLE IV.

Inhibition of Growth of Two Protozoa by Varying Dilutions of Protoanemonin.

Organism	Dilution of Protoanemonin							Media used	Time of reading, days
	200,000	300,000	400,000	500,000	1,600,000	3,200,000	Controls		
Tetrahymena geleii (3 strains)	0	+	+++	+			+++	2% proteose peptone or bactopeptone	2
Tryp. gambiense guinea pig blood	0		++++	+++			++++	Weinman's	5
Tryp. gambiense culture					++++	0	++++	''	5

toanemonin. The experiments, thus, failed to show any inhibitory effect of protoanemonin on either coli or staphylococcus bacteriophage.

Methods and results of testing influenza virus grown in chick embryos. In order to study the action of protoanemonin on the growth of influenza virus in fertile eggs, it was necessary, first, to determine the amount of protoanemonin which could be tolerated by the chick embryo. The tests showed that 0.2 cc of a 1-1000, 1-2000 or 1-4000 dilution of protoanemonin was not toxic when injected 2 days in succession in 11- or 12-day-old fertile eggs and the latter 2 concentrations were harmless to 10-day-old eggs.

The treatment of influenza infected eggs with protoanemonin in dilutions non-injurious to the chick embryo then was attempted. The combination of 1-2000 dilution of protoanemonin and 10^{-5} or 10^{-6} dilution of influenza virus killed the embryo, while the same dilution of protoanemonin with 10^{-7} dilution of virus resulted in a viable embryo with a concentration of virus comparable to that in the control eggs. The growth and titer of the virus in the allantoic fluid was determined by an Hirst[7] agglutination test. The eggs treated with 1-4000 protoanemonin and infected with the same dilutions of virus survived, and it was evident that there was a multiplication of the virus as demonstrated by the agglutination titer. These experiments would indicate that a combination of protoanemonin, non-toxic by itself, and influenza virus in adequate concentration is lethal to the embryo. Furthermore, where conditions are such that the embryo survives there is no evidence of inhibition by protoanemonin of the growth of the virus.

Tissue culture tests. The effect of protoanemonin on tissue cells was tested through the courtesy of Dr. Mary Parshley. Whole thicknesses of chicken skin were planted in chicken plasma diluted one-third with two solutions, one of which was optimal for fibroblasts and the other optimal for epithelial cells. These two solutions contained protoanemonin in concentrations of 1-1 million and 1-5 million. When compared with the

7 Hirst, G. K., *J. Exp. Med.*, 1942, 75, 49.

growth of the controls, it appeared that protoanemonin was toxic to both fibroblasts and epithelial cells in 1-1 million dilution and inhibitory to growth in the 1-5 million dilution. However, it seemed to be less inhibitory for both cells when they were cultivated in the solution which favored the growth of epithelial cells.

Discussion. Protoanemonin is of interest because of its wide range of activity. *In vitro* it inhibits the growth not only of Gram-positive and Gram-negative bacteria but also fungi, two protozoa and fowl epithelial and fibroblastic tissue cells.

The action of protoanemonin on bacteria, fungi and the tetrahymena involves two types of effect, an inhibition of the growth of the microorganisms and an actual killing of the organisms. Only the former effect has been considered in the data presented here. In most cases subcultures from the tubes containing the greatest dilution of protoanemonin inhibiting growth, as reported in the tables, would demonstrate the presence of viable organisms. Indeed, this frequently might be demonstrated by the simple procedure of further incubation of the original tubes. Greater concentrations of the protoanemonin, however, actually kill the organisms. This phase of the action of protoanemonin will be described at a later time.

Another factor under investigation which may influence the action of protoanemonin is the composition of the medium. It may be observed that the bacteria tested for sensitivity to protoanemonin in both meat infusion broth and casein hydrolysate broth were inhibited by greater dilutions of protoanemonin in the latter medium. This influence of medium on the sensitivity of an organism to protoanemonin was apparently unrelated to the relative growth of the organisms in the two broths. *Staphylococcus oxford* H. *Vibrio cholerae* and *Pseudomonas aeruginosa* grew better in the infusion broth, while *Escherichia coli* and *Corynebacterium xerosis* grew as well or better in the casein hydrolysate broth. The casein hydrolysate medium was employed because it is apparent, as seen in the tables, that the greatest dilution of protoanemonin

causing inhibition of growth of a given organism sometimes varied considerably in different tests. It was thought that the introduction of a semi-synthetic medium, more uniform in its composition than meat infusion broth, might prevent this fluctuation. It has continued, however, even when casein hydrolysate broth was used. The explanation for this is not at present available.

Certain mammalian bloods, when added to culture media, cause a decrease in sensitivity to protoanemonin. For example, guinea pig blood is highly inhibitory. This may contribute to the difference in the titer of activity of protoanemonin against *Trypanosome gambiense* in tubes inoculated with infected guinea pig blood and those inoculated from culture media. There also is the possibility that the diverse forms of trypanosomes found under the two conditions of growth contribute to the variation in sensitivity.

The method of testing the sensitivity of the bacteriophages was such that protoanemonin was in contact only with the resting bacteriophage. Experiments to be reported indicate that protoanemonin is relatively harmless to resting bacteria and is antagonistic mainly during the period of active growth. It is therefore possible that the method was not suitable to indicate the effect of this agent on bacteriophage. The antibiotic nature of protoanemonin makes it difficult to evaluate when the organism tested requires a viable and actively growing substrate. Thus the failure to demonstrate inhibition of the influenza virus may have been due to the fact that such small amounts of protoanemonin were tolerated by the chick embryo that the effective concentration of protoanemonin for the virus was not achieved.

Summary. 1. Protoanemonin inhibited the growth of all the aerobic and anaerobic bacteria, the fungi and the protozoa tested. 2. The maximum dilution of protoanemonin which was effective against the bacteria and fungi varied from 1-6000 to 1-300,000. 3. The anti-bacterial activity of protoanemonin was independent of the acidity of the medium, the size of the inoculum and the age of the culture, within the limits tested. 4. The two

protozoa were prevented from growing in dilutions of protoanemonin ranging from 1-200,000 to 1-1,600,000. 5. No inhibition by protoanemonin of the growth of the two bacteriophages and the influenza virus was demonstrable by the techniques employed. 6. A dilution of 1-1,000,000 protoanemonin was toxic for chicken tissue culture epithelial and fibroblastic cells.

15982

Determination of O₂ Capacity on 39.3 Cubic Millimeters of Blood.

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In a recent study of bone marrow blood gases[1] in which the use of minimal sample volumes was imperative, estimation of O_2 capacity was necessary. The current procedure used for such determinations involves aeration of blood after which the O_2 content is determined. This technique has been used by Roughton and Scholander[2] and by Lilienthal and Riley.[3] Preliminary aeration was carried out in a separate vessel with 0.5 or more cc of blood although only 39.3 cmm were used for the O_2 determination. When blood is aerated in a flask or syringe, a certain amount of plasma is lost by evaporation as well as by adherence to the sides of the vessel. Should quantities of blood smaller than 0.5 cc be used, concentration of the red cells becomes greater. To avoid the errors inherent in separate aeration and to reduce the amount of blood required, the micro method of Roughton and Scholander has been modified so that the O_2 capacity can be determined on a total blood sample of only 39.3 cmm.

Apparatus. The Roughton-Scholander syringe and pipette (Roughton and Scholander[2]) are employed. A second mark (designated "upper mark") is scratched on the syringe cup at a distance above the existing one such that the volume of the cup as measured between the two marks is approximately 25 cmm. Since the diameter of most cups is 2.5 mm, the upper mark may be made 5 mm above the first. In the estimation of O_2 capacities exceeding 16 vol % the syringe with the 50 unit capillary is necessary.

Reagents. In addition to those listed by Roughton and Scholander 0.9% NaCl is required.

Sampling blood. From a finger prick or a needle inserted in a vessel, blood is sucked directly into the 39.3 cmm pipette which has been previously flushed with anti-coagulant solution (heparin) and dried in a current of air.

Procedure. 1. The syringe is flushed 3 times with separate portions of saline, emptied, and the cup filled to the lower mark with saline.

2. The pipette is filled to its mark with blood; its tip is passed carefully into the cup containing saline and pressed firmly against the bottom. 3. Blood is sucked into the syringe by a smooth withdrawal of the plunger. Once the pipette is emptied it is quickly removed and the saline in the cup is drawn in after the blood.

4. The blood-saline mixture is lowered far enough into the syringe to permit entry of an air volume of approximately 1.0 cc.

5. The syringe is then rotated for 5 minutes on its long axis with an occasional rotation at right angles to spread a small layer of blood over the inside of the syringe and thus ensure

1 Grant, W. C., and Root, W. S., *Fed. Proc.*, 1947, 6, 114.

2 Roughton, F. J. W., and Scholander, P. F., *J. Biol. Chem.*, 1943, 148, 541.

3 Lilienthal, J. L., Jr., and Riley, R. L., *J. Clin. Invest.*, 1944, 23, 904.

TABLE I.
Comparison of Oxygen Capacities of Blood by
Sendroy Method (1.0 cc) and Micromethod
(39.3 cmm).

Sendroy, Vol. %	Micro, Vol. %	Difference, Vol. %	Hct. (Van Allen), %
20.3	20.2	—0.1	45.9
19.6	19.8	+0.2	44.5
15.2	15.1	—0.1	35.9
16.7	16.8	+0.1	37.3
17.1	17.2	+0.1	40.8
14.5	14.2	—0.3	35.9
12.5	12.8	+0.3	30.8
10.5	10.4	—0.1	24.1
15.8	15.7	—0.1	38.0

uniform exposure of blood to the air. Once a minute during this procedure the air is renewed by alternately running the blood up to the capillary and lowering to the 1.0 cc mark. In certain syringes the formation of transverse blood films makes the expulsion of air without loss of blood difficult. The addition of 2 capillary divisions of caprylic alcohol alleviates this condition.

6. Air is expelled and blood-saline pushed up to the bottom of the cup. The cup is filled to the upper mark with ferricyanide solution, and the solution is then lowered to the bottom of the cup.

7. Two divisions of caprylic alcohol are drawn into the top of the capillary and the cup emptied.

8. Acetate buffer is added to the upper mark and the original Roughton-Scholander[2] (page 545) procedure for O$_2$ content is then followed.

Blank determination. To obtain a blank value for reagents as well as for physically dissolved oxygen of the blood, an analysis is performed exactly as indicated above; but instead of delivering the usual 39.3 cmm of blood, 43.3 cmm of 0.9% NaCl is substituted. For this purpose a mark indicating 43.3 cmm volume, or 110% of the usual 39.3 cmm volume, is scratched on the pipette. Sendroy *et al.*[4] have shown that for practical purposes 1.1 cc of aerated saline solution contains the same volume of physically dissolved O$_2$ as 1.0 cc of blood.

Calculations. Calculation of results follows

that described by Roughton and Scholander.[2]

Aeration of blood. Quite unexpectedly complete saturation of the blood mixture is obtained in only 5 minutes. This is accomplished by the vigorous rotation of the blood with air which is expelled and replaced with fresh air at intervals of a minute, as described above. The ratio of total air to liquid volume is approximately 100 to 1. That a longer time of aeration does not increase the O$_2$ content was demonstrated by noting that, on the same blood, two determinations after 5 minutes aeration showed O$_2$ capacities of 17.0 and 16.8 vol % and two others after 10 minutes of aeration, 16.9 and 16.9 vol %.

Accuracy of method. The O$_2$ capacity as determined on 1.0 cc of blood with the standard method of Sendroy[5] was compared with that measured upon 39.3 cmm of the same blood with the micromethod. These analyses were performed by 2 individuals, one using the Sendroy method and the other the micromethod. The results obtained on 6 dogs are shown in Table I. Hematocrit values measured with Van Allen[6] tubes are included for reference. The agreement between the 2 procedures was good, the maximum difference being 0.3 vol %.

Successive measurements performed on the same sample by the micromethod yielded variations of similar magnitude as demonstrated in the following 2 samples: (a) 15.5, 15.6 vol % and (b) 11.2, 11.4, 11.2 vol %.

Discussion. The Roughton-Scholander micromethod for the measurement of the O$_2$ content of blood has been modified so that O$_2$ capacity can be determined on a total blood sample of 39.3 cmm. Since the blood is both aerated and analyzed in the same apparatus the transfer from aeration vessel to Roughton-Scholander syringe is avoided. The elimination of this step which is required when aeration is carried out in a separate vessel avoids the possibility of analyzing blood which contains a concentration of red cells unlike that obtained from the subject. The method permits the estimation of O$_2$

[4] Sendroy, J., Jr., Dillon, R. T., and Van Slyke, D. D., J. Biol. Chem., 1934, 105, 597.

[5] Sendroy, J., Jr., J. Biol. Chem., 1931, 91, 307.
[6] Van Allen, C. M., J. Lab. and Clin. Med., 1925, 10, 1027.

capacity on blood obtained from a finger prick. It should prove useful where measurements are needed on small animals such as mice or rats or under conditions where venipuncture is difficult or undesirable, as in premature infants.

Summary. The Roughton-Scholander microgasometric procedure has been modified to provide a simple and accurate micromethod for the determination of the O_2 capacity of blood. Only 39.3 cmm are required and an analysis may be completed in 10 to 12 minutes. O_2 capacities obtained by the micromethod agree well with those measured by the Sendroy macromethod, the average difference being ±0.16 vol %.

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Renal Excretion of Mannitol.[*]

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The evidence that the clearance of inulin in man is a measure of the rate of glomerular filtration, although necessarily indirect, seems fairly conclusive.[1] Other studies by Smith and his colleagues[2] have suggested that a number of hexitols (including mannitol) are excreted solely by glomerular filtration. Finally, Gilman has reported[3] that the clearance of thiosulfate is also a measure of the filtration rate.

Recently, a patient was observed in this laboratory whose inulin clearance was found to be 144 ml per minute, but whose mannitol clearance, 3 months later, was 94 ml per minute. Since this patient had no demonstrable kidney disorder and since there was no reason to expect any significant change in renal function during the elapsed period, it appeared likely that inulin and mannitol clearances are not always identical. Further observations on this point, therefore, were undertaken and form the basis of this report.

Some support for the belief that the mannitol clearance is less than that of inulin is obtained from published reports. The most extensive series of observations on the inulin clearance in normals is that of Smith and his colleagues[4] who found the average value of 131 ml per minute with a standard deviation of 21.5 ml per minute in 67 males, and an average of 117, with a standard deviation of 15.6, in 21 females. The findings of most other investigators are in line with those of Smith and are summarized in Table I. Also included are previously unpublished data from this laboratory. The average inulin clearance for the entire group of 133 males is 128 ml per minute. In contrast, the average mannitol clearance in 52 males is 116 ml per minute. The difference between the mean mannitol and inulin clearances among the male patients is statistically significant. There is little difference between the mannitol and inulin clearances among the female patients.

There are but few published data on direct comparisons between mannitol and inulin

1 Smith, H. W., *Physiology of the Kidney*, Oxford University Press, New York, 1937.

2 Smith, W. W., Finkelstein, N., and Smith, H. W., *J. Biol. Chem.*, 1940, **135**, 231.

3 Newman, E. V., Gilman, A., and Philips, F. S., *Bull. Johns Hopkins Hosp.*, 1946, **79**, 229.

4 Smith, H. W., *Lectures on the Kidney*, University of Kansas, Extension Division, 1943.

TABLE I.
Summary of Inulin and Mannitol Clearances in Normal Human Subjects Reported from Various Laboratories. All values are corrected to a surface area of 1.73 sq. m.

Author	Male			Female		
	No. of subjects	Mean clearance ml/min.	Standard deviation	No. of subjects	Mean clearance ml/min.	Standard deviation
Inulin.						
Smith[4]	67	131	21.5	21	117	15.6
This laboratory*	26	126	17.1	7	111	16.5
Friedman[6,7]	16	129	30.6	5	126	14.0
Bradley[12]	8	114	15.0	6	128	19.5
Foa[8]	7	117	30.8			
Miller[9]	5	115	16.4			
Talbott[10]	4	150	35.8			
Total group	133	128	23.5	39	119	16.4
Standard error of the mean: 2.04.						
Mannitol.						
This laboratory*	23	111	17.8	2	108	
Bradley[12]	10	141	43.0	23	124	18.9
Newman†[11]	9	103	15.6			
Klopp[13]	6	117	23.8			
Lauson‡[14]	4	107	25.5	1	125	
Total group	52	116	28.6	26	123	24.5
Standard error of the mean: 3.97.						

Standard error of the difference of the means for males: 4.46, t = 2.69. Probability of such a difference occurring by chance, less than 0.007.
* Includes published[5] and unpublished data.
† Estimated from Fig. 4 of the paper by Newman et al.[11]
‡ Data derived from patients 17-76 days after recovery from shock.

clearances. The average ratio of mannitol to inulin clearance in 8 observations on 6 patients reported in the original paper on mannitol[2] was 0.99 with a range of 0.96 to 1.03. One of these patients had hypertension. 2 were normal ante-partum females, while the remainder had preeclampsia. Eight comparisons were reported[5] among 7 patients with various degrees of renal functional impairment due to glomerulonephritis. The mean ratio was 0.96, with a range of 0.92 to 1.03. Lauson and his colleagues reported[14] a mean ratio of mannitol to inulin clearance ratio of 0.90 among 5 patients suffering from shock. The authors state, however, that they were experiencing minor technical difficulties with the inulin method and that ". . . it appears likely that the average difference of 10% represents a systematic technical error rather than a true difference in clearance." It is to be noted that the majority of the reported comparisons between mannitol and inulin clearances were performed in patients who either did have or probably had some renal abnormality. Three comparisons between the mannitol and creatinine clearances were performed in 2 dogs and are reported in the original paper on mannitol.[2] In one dog, the ratio was 0.98. but both experiments in the other animal yielded a ratio of 0.92.

[5] Earle, D. P., Taggart, J. V., and Shannon, J. A., J. Clin. Invest., 1944, 23, 119.

[6] Friedman, M., Selzer, A., and Rosenblum, H., J. Clin. Invest., 1941, 20, 107.

[7] Friedman, M., Selzer, A., Sugarman, J., and Sokolow, M., Am. J. Med. Sc., 1942, 204, 22.

[8] Foa, P. P., Woods, W. W., Peet, M. M., and Foa, N. L., Arch. Int. Med., 1942, 69, 822.

[9] Miller, B. F., Alving, A. S., and Rubin, J., J. Clin. Invest., 1940, 19, 89.

[10] Talbott, J. H., Pecora, L. J., Melville, R. S., and Consolazio, W. V., J. Clin. Invest., 1942, 21, 107.

[11] Newman, E. V., Bordley, J., and Winternitz, J., Bull. Johns Hopkins Hosp., 1944, 75, 253.

[12] Bradley, S. E., personal communication.

[13] Klopp, C., Young, N. F., and Taylor, H. C., J. Clin. Invest., 1945, 24, 117, 189.

[14] Lauson, H. D., Bradley, S. E., and Cournand, A., J. Clin. Invest., 1944, 23, 381.

Methods. All inulin and mannitol† clearances reported in this paper for man were performed in patients with no demonstrable renal abnormalities, and represent the average of at least 3 consecutive periods. Urine was collected by a many-eyed catheter, with bladder rinses with sterile water, followed by an injection of air. Creatinine clearance was used as the standard of comparison in similar studies in the dog.

Chemical. Inulin was measured by Harrison's modification[15] of the Alving method.[16] The urines as well as the plasmas were yeasted so that any possible adsorption of inulin on yeast would not constitute an error in the determination of the inulin clearance. Mannitol was measured in some instances by a modification[5] of Smith's technique[17] and, in all experiments, by the chromotropic acid method of Corcoran and Page.[18] Creatinine was estimated by a modification of the Folin procedure.[19]

Since much of this study was based on simultaneous mannitol and inulin clearances, it was important to investigate any possible interference of one substance with the chemical determination of the other. In these experiments, the concentration of substance run through its specific analysis was in the range utilized in the clearance studies, while the possible interfering substance was added both at the concentration used in the clearances and in excess. Each study was done in duplicate. The results of the examination of the inulin procedure will be presented in detail.

One mg percent and 10 mg % solutions

of mannitol reacted with diphenylamine reagent as in the inulin procedure gave readings identical to water treated in the same manner. A 1 mg % standard inulin solution gave the same result when run through the reaction above or in the presence of 1 to 10 mg % mannitol. Five, 10, and 15 mg % inulin standards, yeasted and then run through the diphenylamine reaction, resulted in a mean recovery of 99.5% compared to aqueous non-yeasted solutions, while the mean recovery for a similar series of inulin standards was 99.6% in the presence of 150 mg % mannitol. The mean recovery of 10, 15, and 25 mg % inulin solutions when added to normal plasma was 98.8% and when added to plasma containing 100 mg % mannitol, was 97.6%. The addition of 150 mg % mannitol to normal plasma had no effect on the apparent inulin blank, nor did 150 mg % mannitol solution change the apparent concentration of inulin in the plasma of a patient receiving this material. Finally, the presence or absence of mannitol in urine containing inulin made no difference in the result obtained with the inulin reaction. The urines in these studies were yeasted.

Similarly, the presence of inulin did not alter the analysis of mannitol by the chromotropic acid method, either in aqueous solutions, in plasma, or in urine, the recovery of mannitol in all these circumstances ranging from *99 to 101% whether or not inulin was present.* Inulin also did not alter the apparent plasma blank for mannitol. Inulin did not interfere with the analysis of mannitol by the titration method, either in aqueous solutions or after yeasting.

Results. Simultaneous inulin and mannitol clearances were performed as 9 separate experiments in 8 normal subjects. In each instance, the mannitol clearance was less than that of inulin, the mannitol to inulin clearance ratio ranging from 0.79 to 0.96. The mean inulin clearance for the group was 125 ml per minute, the mean mannitol clearance was 109, yielding a mean clearance ratio of 0.87. These data are summarized in Table II. Mannitol was measured by Smith's titration method as well as by the chromotropic acid method

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† The mannitol used in these studies was obtained through the courtesy of Dr. Karl Beyer of Sharp & Dohme, Inc.

15 Harrison, H. E., PROC. SOC. EXP. BIOL. AND MED., 1942, 49, 111.

16 Alving, A. S., Rubin, J., and Miller, B. F., J. Biol. Chem., 1939, 127, 609.

17 Goldring, W., and Chasis, H., *Hypertension and Hypertensive Disease,* Commonwealth Press, 1944.

18 Corcoran, A. C., and Page, I. H., *Fed. Proc.,* 1946, 5, 130.

19 Folin, O., and Wu, H., *J. Biol. Chem.,* 1919, 38, 81.

TABLE II.
Comparison of Inulin and Mannitol Clearances in Males.
All values represent the average of 3 or more consecutive periods and are corrected to a surface area of 1.73 sq. m.

Patient	Age	Date	Inulin clearance ml/min	Mannitol clearance ml/min	Clearance ratio mannitol/ inulin	Control inulin clearance ml/min	Decrease after mannitol %	Control mannitol clearance ml/min	Decrease after inulin - %
Las	46	1/2	142*						
		2/26	141*						
		4/11	139	118	0.85	158	12.0		
Sol	42	4/9	105	101	0.96	124	14.8		
McC	31	4/18	155	140	0.90	134	11.5		
War	34	3/26	138	109	0.79				
		4/15	116	110	0.95	122	6.1		
Alv.	46	4/23	126	112	0.89			122	7.9
Vad	39	4/28	156	123	0.79			134	8.4
		5/15	144*						
Mor	40	3/24	116*						
		4/7	96.5	84.8	0.87				
Seh	48	3/19	96.0	94.0	0.88				
Avg			125	109	0.87	135	11.1		

* Not included in average.

in 2 of the experiments. The results were identical for both methods. In 4 of the studies shown in Table II, control inulin clearances were measured prior to the infusion of mannitol. The inulin clearance was lower after mannitol in each instance, the average depression being 11.1%. The reverse experiment, wherein control mannitol clearances were obtained before the administration of inulin, resulted in smaller and transient depressions in mannitol clearance. The average decreases in mannitol clearance were 7 and 8%, but in each instance the mannitol clearance had almost returned to control values within one hour after the mannitol injection. In several patients (Las, Mor, and Vad, Table II), inulin clearances alone were performed on another date. In each instance, the inulin clearance done separately more closely approximated the inulin than the mannitol value when the two were done simultaneously.

The creatinine clearance is generally believed to be a measure of glomerular filtration rate in the dog. For this reason, mannitol and inulin clearances were compared separately to that of creatinine in 3 unanesthetized female dogs. These data are summarized in Table III. The mean inulin to creatinine clearance ratio was 1.01, while the mannitol to creatinine clearance ratio in the same 3 dogs was 0.88.

Although metabolic alteration of a compound does not preclude its use as a measure of glomerular filtration rate by urine collection techniques, the application of the constant infusion technique[20] requires that there be no metabolic alteration of the substance. If the renal clearance of a substance, as measured by urine collections, is less than the clearance as measured by the infusion technique, it may be concluded that some of the substance is metabolized. The ratios of the clearances of mannitol as measured by the two techniques ranged from 0.75 to 1.00, with an average of 0.88. The mean urine to infusion pump clearance for inulin on 31 subjects yielded a ratio of 1.02.[21] In 3 normal subjects, 80, 85, and 90% of administered mannitol was recovered from the urine collected for 24 hours after the dose. Smith and his co-workers[2] likewise found 81 and 89% recovery of mannitol during the 10½-hour period after the dose, but in similar experiments with inulin, recovered 95 and 97% in the same 2 patients.

Discussion. The mannitol clearance has been found to be consistently lower than the simultaneously measured inulin clearance in 9 patients with no demonstrable renal dis-

20 Earle, D. P., and Berliner, R. W., Proc. Soc. Exp. Biol. and Med., 1946, 62, 262.
21 Unpublished data.

Methods. All inulin and mannitol† clearances reported in this paper for man were performed in patients with no demonstrable renal abnormalities, and represent the average of at least 3 consecutive periods. Urine was collected by a many-eyed catheter, with bladder rinses with sterile water, followed by an injection of air. Creatinine clearance was used as the standard of comparison in similar studies in the dog.

Chemical. Inulin was measured by Harrison's modification[15] of the Alving method.[16] The urines as well as the plasmas were yeasted so that any possible adsorption of inulin on yeast would not constitute an error in the determination of the inulin clearance. Mannitol was measured in some instances by a modification[5] of Smith's technique[17] and, in all experiments, by the chromotropic acid method of Corcoran and Page.[18] Creatinine was estimated by a modification of the Folin procedure.[19]

Since much of this study was based on simultaneous mannitol and inulin clearances, it was important to investigate any possible interference of one substance with the chemical determination of the other. In these experiments, the concentration of substance run through its specific analysis was in the range utilized in the clearance studies, while the possible interfering substance was added both at the concentration used in the clearances and in excess. Each study was done in duplicate. The results of the examination of the inulin procedure will be presented in detail.

One mg percent and 10 mg % solutions

of mannitol reacted with diphenylamine reagent as in the inulin procedure gave readings identical to water treated in the same manner. A 1 mg % standard inulin solution gave the same result when run through the reaction above or in the presence of 1 to 10 mg % mannitol. Five, 10, and 15 mg % inulin standards, yeasted and then run through the diphenylamine reaction, resulted in a mean recovery of 99.5% compared to aqueous non-yeasted solutions, while the mean recovery for a similar series of inulin standards was 99.6% in the presence of 150 mg % mannitol. The mean recovery of 10, 15, and 25 mg % inulin solutions when added to normal plasma was 98.8% and when added to plasma containing 100 mg % mannitol, was 97.6%. The addition of 150 mg % mannitol to normal plasma had no effect on the apparent inulin blank, nor did 150 mg % mannitol solution change the apparent concentration of inulin in the plasma of a patient receiving this material. Finally, the presence or absence of mannitol in urine containing inulin made no difference in the result obtained with the inulin reaction. The urines in these studies were yeasted.

Similarly, the presence of inulin did not alter the analysis of mannitol by the chromotropic acid method, either in aqueous solutions, in plasma, or in urine, the recovery of mannitol in all these circumstances ranging from 99 to 101% whether or not inulin was present. Inulin also did not alter the apparent plasma blank for mannitol. Inulin did not interfere with the analysis of mannitol by the titration method, either in aqueous solutions or after yeasting.

Results. Simultaneous inulin and mannitol clearances were performed as 9 separate experiments in 8 normal subjects. In each instance, the mannitol clearance was less than that of inulin, the mannitol to inulin clearance ratio ranging from 0.79 to 0.96. The mean inulin clearance for the group was 125 ml per minute, the mean mannitol clearance was 109, yielding a mean clearance ratio of 0.87. These data are summarized in Table II. Mannitol was measured by Smith's titration method as well as by the chromotropic acid method

† The mannitol used in these studies was obtained through the courtesy of Dr. Karl Beyer of Sharp & Dohme, Inc.

15 Harrison, H. E., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 111.

16 Alving, A. S., Rubin, J., and Miller, B. F., *J. Biol. Chem.*, 1939, **127**, 609.

17 Goldring, W., and Chasis, H., *Hypertension and Hypertensive Disease*, Commonwealth Press, 1944.

18 Corcoran, A. C., and Page, I. H., *Fed. Proc.*, 1946, **5**, 130.

19 Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, **38**, 81.

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Dental Caries in the Cotton Rat IX. Effect of Milk Rations[*]

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Investigations in this laboratory on the experimental production of dental caries in the cotton rat showed that the molars of this animal were highly susceptible to decay when the rat was fed a dry ration high in fermentable sugar.[1,2] In contrast to this, animals fed on a diet consisting of liquid whole milk were found to be completely free of caries.[3] The consumption of a dry ration approximating milk solids in composition resulted in a low incidence and extent of carious lesions. although protection was not complete. as it was in the case of the liquid milk. The protection afforded by milk is as great as, or greater than. that found with any other ration. and further study of this effect therefore seemed important. Two approaches were made: (1) fermentable sugars known to be highly cariogenic when fed as the carbohydrate portion of dry rations were added to the liquid milk diet: and (2) supplements of milk were given to animals being fed the cariogenic control ration.

Experimental. The cotton rats used in these experiments were raised in our own stock colony and placed on experiment at weaning (20-25 g). Animals from each litter were distributed as equally as possible among the control and experimental groups, since susceptibility to dental caries has been found to vary among different litters.[4] The animals were kept on experiment for 14 weeks. during which time their weights were recorded at bi-weekly intervals. At the end of the experimental period, the animals were killed. and the incidence and extent of carious lesions in each animal were evaluated by the method of Shaw and his associates.[1,5]

The control animals were fed a sucrose ration (S02) previously found to be highly cariogenic.[†] The liquid whole milk ration was fortified with minerals (iron, copper and manganese) and with 1:20 liver extract as described by Schweigert and co-workers.[3] The carbohydrate (sucrose, glucose or dextri-maltose) was added to the milk at a 5% or 10% level. This. together with liver extract. was homogenized into the milk in a Waring blendor. These diets were fed *ad libitum.* In one group milk was fed in addition to the S02 ration. In this case the milk was fed *ad libitum,* but the intake of ration S02 was limited to 63% of that of the controls (S02 ration only) in order to induce greater consumption of the milk. The milk consumed constituted roughly one-third of

1 Shaw, J. H., Schweigert, B. S., McIntire, J. M.. Elvehjem. C. A. and Phillips, P. H., *J. Nutr.,* 1944, 28, 333.

2 Schweigert, B. S., Shaw, J. H., Phillips, P. H., and Elvehjem. C. A., *J. Nutr.,* 1945, 29, 405.

3 Schweigert, B. S., Shaw. J. H., Zepplin, M.. and Elvehjem, C. A., *J. Nutr.,* 1946, 31, 439.

4 Schweigert, B. S., Shaw, J. H., Elvehjem, C. A., and Phillips, P. H., Proc. Soc. Exp. Biol. and Med., 1945, 59, 44.

5 Shaw, J. H., Schweigert, B. S., Elvehjem, C. A., and Phillips, P. H., *J. Dent. Res.,* 1944, 23, 417.

† This ration consisted of 67% sucrose, 24% casein, 5% corn oil and 4% salts IV. Four per cent of 1:20 liver extract was added at the expense of the entire ration. Adequate quantities of the B vitamins[6] were added to the ration, and each rat received one drop of halibut liver oil per week.

6 McIntire, J. M.. Schweigert, B. S., and Elvehjem. C. A., *J. Nutr.,* 1944, 27, 1.

TABLE III.
Comparison of Inulin and Mannitol to Creatinine Clearances in Dogs.
All values represent the average of 3 or more consecutive periods.

Dog No.	Clearances, ml/min			Ratios	
	Creatinine	Inulin	Mannitol	Inulin/creatinine	Mannitol/creatinine
1	52.0	53.0		1.02	
	66.2		59.5		0.90
2	51.5	52.2		1.01	
	39.7		33.9		0.85
3	69.3	69.2		1.00	
	62.4		55.8		0.89
				Avg 1.01	0.88

orders. In addition, the mannitol clearance is less than the simultaneous creatinine clearance in the dog, while the inulin clearance has been consistently equal to the creatinine clearance. Recently, Smith and his co-workers[22] have found that mannitol clearances are less than creatinine in the dog. Corcoran[23] has found an average mannitol/inulin clearance ratio of 0.902 with a standard error of 0.017 for 42 observations in dogs and human beings. The mannitol/creatinine clearance ratio in the dogs averaged 0.872 with a standard error of 0.013, and the average mannitol/thiosulfate clearance ratio in dogs was 0.89. Hoobler[24] observed an average mannitol/thiosulfate clearance ratio of 0.89 with a standard deviation of 0.07 in 52 observations in 14 patients.

It also appears that the administration of mannitol by intravenous infusion results in a small but significant decrease in the inulin clearance and presumably in the glomerular filtration rate. This action is not necessarily due to the mannitol itself, but may be the result of other substances contained in the mannitol preparation and is presumably due to alterations in the glomerular hemodynamics.

The evidence summarized by Smith[1] for the belief that inulin is a measure of glomerular filtration rate in man is very strong. This belief is strengthened by the identity between inulin and creatinine clearances in the dog, it being generally believed that creatinine clearance is a measure of filtration rate in this species. For these reasons, it seems probable that the discrepancies between mannitol and inulin clearances are due to reabsorption of a small proportion of filtered mannitol by the renal tubules.

It appears likely, therefore, that mannitol cannot be used as a precise measure of glomerular filtration rate in man. Since some mannitol undergoes metabolic alteration, it certainly cannot be utilized for the measurement of filtration rate by the infusion pump technique. It should be noted that mannitol is an excellent osmotic diuretic, and that although thiosulfate appears to be a measure of filtration rate, it in itself is an electrolyte and in its excretion must carry with it its equivalent cation. For these reasons, neither mannitol nor thiosulfate is ideal for the measurement of filtration rate during the course of experiments directed toward the study of water or electrolyte excretion. Inulin, therefore, remains the substance of choice for the measurement of glomerular filtration rate in man under the widest variety of circumstances.

Conclusions. 1. The renal clearance of mannitol is frequently less than the simultaneous clearance of inulin in man. 2. The intravenous injection of mannitol depresses the inulin clearance in man. 3. The clearance of inulin is equal to that of creatinine in the dog, but the mannitol clearance is usually less than that of creatinine. 4. Mannitol undergoes metabolic alteration in man, and therefore, cannot be utilized in the infusion pump technique for measuring the rate of glomerular filtration. 5. Inulin appears to be the most generally useful substance for the measurement of glomerular filtration in man.

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22 Brode, J., *et al.*, to be published.
23 Corcoran, A. C., personal communication.
24 Hoobler, S. W., personal communication.

averages being 14 and 25+ for the experimental animals as compared with 31 and 78+ for the controls. Thus, the replacement of approximately one-third of the caloric intake by liquid milk resulted in a lowered caries score.

Discussion. From these data it appears that a diet of liquid whole milk is protective against dental caries in the cotton rat. Even in the presence of fairly high levels of fermentable sugars, it exerts a protective action. The protection is less, but evident, when a cariogenic dry ration is fed in addition to the milk. Since it is probable that such protection is the result of a combination of factors, it is difficult to speculate on the mechanism involved.

On a percentage basis the composition of these milk diets, even with the carbohydrate additions, resembles that of a "medium fat" ration used in earlier experiments.[3,7] In previous work all dry rations with this medium fat level have given partial protection against caries, although scores have not usually been as low as they were on these milk diets.

The kind of carbohydrate may also have been important. The carbohydrate portion of these diets was at least partially lactose, which appears from our preliminary evidence to be somewhat less cariogenic than the more fermentable sugars. The effects of the various fermentable sugars, however, seem to be similar. The caries scores on the milk plus carbohydrate rations showed considerable variation; there appeared to be no consistent trend, and overlapping of individual scores occurred. In dry rations of the medium fat level, these same sugars—sucrose, glucose and dextri-maltose—showed no significant differences in caries production.[7]

The differences in caries scores between the rats fed only a liquid diet and those receiving dry ration in addition suggests that the physical state of the ration may play a considerable role in the protective action. In the liquid diets carbohydrates were consumed in the dissolved state, and it is possible to attribute the protection exhibited to a lack of mastication or to the fact that the carbohydrates probably remained in the mouth for a comparatively short time. The lower growth rates of the animals on the liquid diets, as compared with their controls, indicate that a difference in caloric intake may also have been a contributing factor.

In this connection it is interesting to note the possible effect of the condition of the fat, which exists in relatively free form in liquid milk. Rosebury and Karshan[8] found that free oil or fat in the diet was effective in reducing the caries index in white rats. They postulated that lipids may form a mechanical coating on the tooth surface or on the particles of food, thus protecting against bacterial action, an idea also suggested by McCollum.[9]

As yet, inadequate investigation has been made of the effect of milk on the growth and activity of microorganisms associated with dental decay, and an inhibitory effect through this channel might be possible. Other conditions of the oral environment, such as pH, might also play a role. Further studies are in progress.

Summary. Data on growth and caries indices of cotton rats fed various milk rations were secured from these experiments.

The data indicate that milk is protective against dental caries in the cotton rat. Previous findings of zero scores in animals fed only liquid milk were confirmed. Animals receiving milk to which sucrose, glucose or dextri-maltose had been added exhibited low caries scores as compared with controls on a cariogenic dry ration. These sugars will produce caries when fed in dry rations. The caries indices of animals receiving approximately one-third of their caloric intake as liquid milk and the remainder as the cariogenic ration 802 were less by 50% than those of litter-mate controls not receiving milk.

[7] Schweigert, B. S., Potts, E., Shaw, J. H., Zepplin, M., and Phillips, P. H., *J. Nutr.,* 1946, 32, 405.

[8] Rosebury, T., and Karshan, M., *J. Dent. Res.,* 1939, 18, 189.

[9] McCollum, E. V., Orent-Keiles, E., and Day, H. G., *The Newer Knowledge of Nutrition,* 5th ed., Macmillan, New York, 1939.

TABLE I.

Effect of Various Milk Rations on Growth Rate and on Incidence and Extent of Carious Lesions in the Cotton Rat.

Ration	No. of pairs	Avg weekly gain		Avg incidence	Avg extent
		6 wks	14 wks		
802*	15	10.1	7.2	29	75+
milk		7.3	5.3	0	0†
802	3	8.4	5.5	30	76+
milk + 5%. sucrose		6.6	4.8	0	0
802*	11	9.5	6.5	32	82+
milk + 10% sucrose		7.4	5.5	4	7+
802	3	11.6	7.8	34	109+
milk + 5% dextrimaltose		10.3	7.2	1	1+
802	7	11.1	8.0	24	50+
milk + 10% dextrimaltose		8.5	7.4	0	0
802*	13	9.0	6.7	27	68+
milk + 10% glucose		7.3	5.8	6	10+
802	9	9.5	7.8	31	78+
802 + milk		6.7	7.7	14	25+

* These groups included one animal which died and was evaluated at 12 weeks instead of at 14 weeks.

† One of these 15 animals had a 1 — 1+ lesion.

the daily caloric intake of the animals in this group.

Results. In Table I the data from the animals on each experimental ration are compared with those from their respective littermate controls. The control and experimental animals from each litter were paired so that averages represented equal numbers of animals.

Growth rates were calculated as average weekly gain for the first 6 weeks and for the total 14 weeks on experiment. Since the two sexes have been found to grow at different rates, all figures were calculated as growth of males in order to make them comparable. This was formerly done by increasing the values for the females by 25%;[2] but analysis of a larger volume of data now at our disposal has shown that more accurate factors are 20% at 6 weeks and 15% for the total 14 weeks. Accordingly, the gains of females were increased by these percentages for the respective periods. From the data it is evident that the animals fed on milk alone grew less than those receiving the control ration (802). Growth was somewhat better when supplemental carbohydrates were added to

the milk, although it was still below that of the control animals. The animals fed ration 802 plus milk grew slowly during the first weeks of the experiment, but the average weekly gain for the total 14 weeks was very nearly the same as that for the controls receiving only the dry ration.

Table I also gives the data on incidence and extent of carious lesions. The comparison of average scores for the experimental animals with those for litter-mate controls receiving the cariogenic ration (802) indicates the degree of protection afforded by each ration. The scores of animals on the milk diets were low in comparison with the high incidence and extent of lesions found in animals fed the control ration. In agreement with previous findings, animals fed only liquid milk were free of caries (except one animal, which developed a small lesion— 1+). The caries indices of animals receiving milk with added carbohydrate varied considerably, ranging from zero to less than one-fourth the average for litter-mate controls. In the case of the animals fed milk plus ration 802, the milk appears to have reduced the caries scores by more than 50%, the
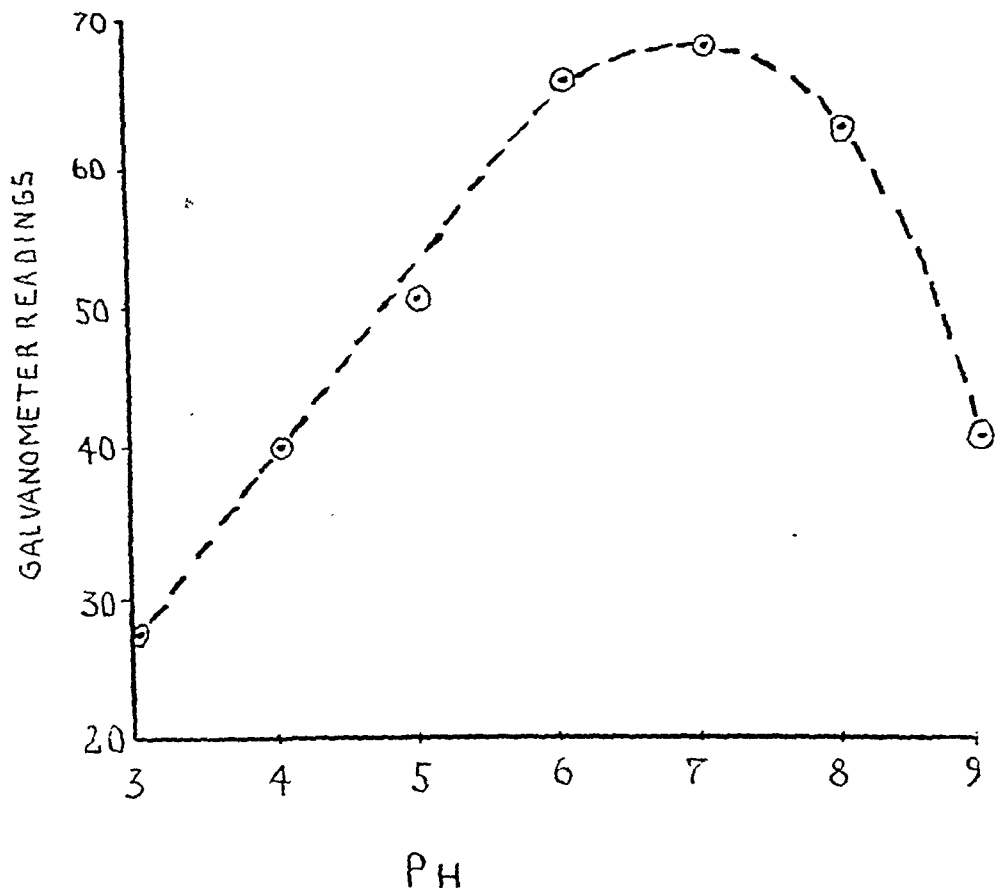
Fig. 1
Relationship of the enzyme activity to pH

II. *Measurement of activity of the bacterial suspension.* A series of tubes was prepared, each containing 5 ml of a standard solution of nicotinic acid (10 μg per ml), 0.5 ml of 0.2 M phosphate buffer of pH 6.0, and 0.05 ml of bacterial suspension. These were allowed to stand at room temperature for 10 to 30 min. and the enzyme was then inactivated by immersion in a water bath at 90°C for 5 min. The tubes were centrifuged for 15 min. at 10,000 r.p.m. and 5 ml of the clear supernatant fluid were analyzed for nicotinic acid by a colorimetric method§ employing cyanogen bromide and p-phenylenediamine. The intensity of the yellow color was measured in a Coleman spectrophotometer at a wave-length of 420 mμ.

§ The procedure used for the estimation of nicotinic acid will shortly be published elsewhere.

Experimental. I. *Activity of the culture.* It was found that 50 μg of nicotinic acid were destroyed in 15 min. at room temperature by 0.05 ml of the bacterial suspension. Under the same conditions there was no appreciable decomposition of nicotinamide.

II. *Effect of pH.* Aliquots of the substrate were adjusted to pH values ranging from 3 to 9. These were inoculated and the amount of nicotinic acid remaining was determined. The results shown in Fig. 1 indicate that the optimum pH was about 7.0.

III. *Attempt to obtain a cell-free enzyme.* Freezing and thawing a sample of the bacterial suspension 8 times in 24 hours destroyed 66% of the normal activity, as is shown in Table I. Experiments are now in progress to obtain cell-free enzymes by other methods.

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Nicotinic Acid Oxidation in *Pseudomonas fluorescens.*[*]

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In the determination of nicotinic acid in blood, Allinson[1] first employed a microorganism as the source of an enzyme which specifically destroyed nicotinic acid. The microorganism used was the NC (neutral culture) isolated from soil by Dubos and Miller.[2] Later Koser and Baird[3] made an extensive investigation of bacteria which destroy nicotinic acid during cell multiplication. They found that bacteria of the *Pseudomonas fluorescens* and *Serratia marcescens* groups grew on a synthetic medium in which nicotinic acid was the only organic compound and that they failed to grow when nicotinic acid was replaced by the isomers, isonicotinic acid and picolinic acid.

The nature and mode of action of the bacterial enzyme have not yet been elucidated. The experiments recorded below show that in the case of *Pseudomonas fluorescens* an oxidative mechanism is involved in the enzymic decomposition of nicotinic acid.

Materials and Methods. I. *Culture:* Preliminary tests with strains 1, 2, 3, 4, 12 and 30[‡] showed that the latter 2 were most active

in decomposing nicotinic acid. *Serratia marcescens* took 7 to 8 days to destroy as much nicotinic acid as was destroyed by *Pseudomonas fluorescens* in 36 hours. The growth of the NC organism was unsatisfactory when the medium was prepared with local tap water or artificial tap water.[4] Since *Pseudomonas fluorescens* 30 was most active and its mineral requirements were easily met by tap water or distilled water plus magnesium sulphate, it was chosen as the test organism. It was grown in media composed of: 2 g Na_2HPO_4; 1.5 g KH_2PO_4; 5 g NaCl; 0.1 g $MgSO_4$; and 2 g nicotinic acid in 1 liter distilled water. The pH was adjusted with sodium hydroxide to 6.8 to 6.9. The temperature was 27°C. The bacteria became well adapted to the medium by repeated transfer of 24 hour cultures. Stock cultures were maintained on agar slopes of nicotinic acid medium and no loss of activity was evident after 6 months. The agar cultures had a bright green color after 18 to 24 hours, which turned to reddish-brown after 48 hours.

To prepare suspensions of the *resting bacteria* transfers were made from the nicotinic acid slopes to 50 ml of medium in 250 ml Erlenmeyer flasks. The flasks were shaken for 24 hours at 25°C after which 10 ml were transferred to 500 ml of medium in a 2-liter flask, the latter being shaken again for 18 to 24 hours. The cells were then separated by centrifuging, washed twice with M/50 phosphate buffer of the pH 7.0, and finally suspended in a volume of sterile water equal to 1/20th the volume of the culture medium. This suspension was stored at 5°C for over 2 months without loss of activity.

1 Allinson, C. M. J., J. Biol. Chem., 1943, 147, 785.

2 Dubos, R., and Miller, B. F., J. Biol. Chem., 1937, 121, 429.

3 Koser, S. A., and Baird, G. R., J. Infect. Dis., 1944, 75, 250.

4 Miller, B. F., Allinson, M. J. C., and Baker, Z., J. Biol. Chem., 1939, 130, 383.

nicotinic acid. In a similar experiment 0.0475 micromoles of nicotinic acid were destroyed and 0.13 micromoles of oxygen were taken up. This corresponds to 2.7 moles of oxygen per mole of nicotinic acid.

Sodium azide and hydroxylamine inhibited the oxidation drastically, as is seen in Fig. 2. The manometric measurements were verified with the colorimetric method. In another test, the detergent, lauryl pyridinium chloride‖ in a concentration of 0.0013%, brought about complete inhibition. Octyl alcohol and toluene inhibited the oxidation 68 and 80%, respectively, as indicated with the colorimetric method. There was no oxygen uptake with nicotinamide as substrate.

Summary and Conclusion. The destruction

‖ We wish to thank the Hooker Electrochemical Corporation, Niagara Falls, N.Y., for a sample of lauryl pyridinium chloride.

of nicotinic acid by *Pseudomonas fluorescens,* previously described by Koser and Baird[3] was found to be an enzymic oxidation. Utilization of nicotinic acid is accompanied by oxygen uptake at a ratio of about 3 oxygen to 1 nicotinic acid. Nicotinamide is not attacked by the oxidase under our experimental conditions. Two types of inhibitors interfere with the enzymatic destruction of nicotinic acid: inhibitors of heavy metal enzymes, such as sodium azide and hydroxylamine, and surface active agents, such as octyl alcohol, toluene and lauryl pyridinium chloride. The rate of oxygen consumption indicates a destruction of the nicotinic acid molecule. Since nicotinic acid serves as a substrate and not as a co-enzyme it should be noted that this reaction sets it apart from its known function in the co-factors.

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Effect of Age in Guinea Pig on Local Passive Sensitization and Skin Reactions Toward Histamine.

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Young guinea pigs are universally accepted as being more suitable for the study of anaphylactic reactions than old animals. Thomsen,[1] who confirmed the work of others, found old guinea pigs more difficult to sensitize than young guinea pigs. He also studied the effect of age on the passive sensitivity developed after injection of anti-horse rabbit serum. Sensitivity was measured by injecting the antigen intravenously. His results showed that the young guinea pigs became more sensitive than the old guinea pigs.

To determine whether age had a similar effect on the production of local anaphylaxis, a comparison was made of the ability to transfer sensitivity passively to the skin of old

[1] Thomsen, Olof, *Z. f. Immunitats. forsch.,* 1917, 26, 213.

and of young animals. The method recently described by Chase[2] for producing local passive sensitization in the skin of the guinea pig was used for making these comparisons.

Since a tissue liberation of histamine or histamine-like substance is thought to occur during anaphylactic reactions,[3,4] the response to intradermal injection of this substance in young and in old guinea pigs was studied.

Material. All of the animals were albinos and were obtained from a single breeding colony. Animals of both sexes were used. The old animals were 2 to 4 years and the young

[2] Chase, M. W., PROC. SOC. EXP. BIOL. AND MED., 1943, 52, 238.

[3] Dale, H. H., and Laidlaw, P. P., *J. Physiol.,* 1910, 41, 318.

[4] Lewis, T., *Brit. M. J.,* 1926, 2, 61.

Nicotinic acid ○ — — —
Nicotinic acid, sodium azide △ · · · · · ·
Nicotinic acid, hydroxylamine hydrochloride □ — · — · —

FIG. 2.
Oxygen uptake of *Ps. fluorescens* 30 with nicotinic acid as influenced by sodium azide and hydroxylamine hydrochloride (M/60). Temperature 27°.

TABLE I.
Effect of Alternative Freezing and Thawing on Enzyme Activity.

Time in min.	μg nicotinic acid destroyed by	
	(1) normal cells	(2) treated cells
5	29.6	5.4
10	47.0	9.9
15	50	17.0
30	—	22.3

IV. *Mode of destruction of nicotinic acid.* Warburg experiments showed that the destruction of nicotinic acid was accompanied by oxygen consumption. There was a decrease in the oxygen consumption when the amount of the substrate was decreased. The experiments were conducted at 27°C with M/15 phosphate buffer of pH 7 as medium. The total volume of liquid in the vessels was 2.3 ml; 0.3 ml of bacterial suspension were employed. The center well contained 0.2 ml of 50% KOH.

A nicotinic acid solution containing 615 μg was tipped into the main vessel from one side arm after equilibrium had been reached. After 75 minutes the oxygen uptake was determined and the reaction was stopped by the addition of 0.5 ml of concentrated sulphuric acid from the second side arm. The residual nicotinic acid was determined colorimetrically. For the oxidation of 0.475 micromoles of nicotinic acid 1.42 micromoles were required, *i. e.* 3 moles of oxygen per mole of

or young adult animals were 4 to 10 weeks of age.

Anti-azoovalbumin guinea pig and anti-horse guinea pig serum were the antiserums employed. The method described by Heidelberger, Kendall, and SooHoo[5] was followed for preparing the azoovalbumin. The anti-azoovalbumin was prepared by injecting 50 mg of the antigen intraabdominally into old guinea pigs. The animals were bled 44 days after the sensitizing dose of antigen was given. The anti-horse guinea pig serum was prepared by injecting 0.01 cc of horse serum intraabdominally into 600 g guinea pigs. These animals were bled 21 days after administration of the antigen.

The histamine dihydrochloride solutions were freshly prepared in saline just before use.

Experimental. The antiserums were injected into the closely clipped skin of the backs of both the young and the old animals. After varying intervals of time the corresponding antigens were injected. In experiments 1, 2, 3, and 4 (Table I) these antigens were administered intraabdominally. The dose of azoovalbumin administered to the young animals was 126 mg. The old animals received 189 mg. The dose of horse serum injected was 0.15 to 0.2 cc for each 100 g of body weight. The reactions were measured 15, 30, 45, 60, 90, and 120 minutes later. In most instances they were fully developed or reached their maximum intensity in 60 minutes; consequently, the mean diameters of these 60-minute values are the only measurements given. In agreement with Chase, reactions were not elicited in all of the young animals. The data given in Table I show that the skin of young guinea pigs is more easily sensitized than the skin of old guinea pigs.

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Exp. No.	No. of animals	Mean wt	Age	Dose of anti-protein serum	Latent period before injecting antigen, hr	Mean dose of antigen inj. intraabdom.	No. with positive reactions	% positive	Mean dimensions of reactions in mm	System
1	12	553	10 wks	0.1 cc undil. serum	24	126 mg	8	67	21.6x24.1x1.1	Azoovalbumin-anti-azoovalbumin
	12	960	2-4 yrs	,,	24	189 ,,	0	0	0	,,
2	5	487	8 wks	0.05 cc 1:4 dilution	24	1.0 cc	5	100	17.8x18.4x1.3	Horse serum-anti-horse serum
3	5	1159	2-4 yrs	,,	24	2.3 ,,	1	20	8.0x11.0 f*	,,
	10	948	2-4 ,,	,,	48	1.6 ,,	2	20	8.5x10.0 f	,,
	5	477	8 wks	,,	96	1.0 ,,	2	40	11.0x12.0 f	,,
	5	1158	2-4 yrs	,,	96	2.3 ,,	1	20	8.0x8.0 f	,,
4	5	398	6 wks	0.1 cc 1:2 dilution	24	0.6 ,,	4	80	23.8x26.0x1.5	,,
	5	1066	2-4 yrs	,,	24	1.7 ,,	1	20	18.0x22.0x0.5	,,
5	5	317	5 wks	,,	24	intradermally 0.1 cc undil. horse serum	5	100	normal site 21.0x22.8x2.0 sensitized site† 36.0x40.5x5.0	,,
	10	1193	2-4 yrs	,,	24	,,	3	30	normal site 16.0x19.3x3.3 sensitized site 20.3x24.3x1.0	,,

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				Histamine	Saline	
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10	375	6 wk	1:100,000	17.3x19.6	13.4x15.4	1.6
10	1080	2-4 yr	,,	16.6x17.7	12.5x14.5	1.6
5	421	7 wk	1:1,000,000	13.8x15.8	13.6x15.4	1.0
5	1145	2-4 yr	,,	14.6x15.2	13.8x15.0	1.1

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Discussion. The foregoing experiments show that there is a marked difference in the ability of anti-protein serums to sensitize the skin of old and of young guinea pigs.

The reactions in the two age groups toward histamine were studied to determine whether a weaker response toward histamine could account for the difference in reactivity. The results show that both old and young guinea pigs react in the same degree. It is of interest to note that Lamson and Pope[6] and Darsie,

et al.[7] were unable to produce reactions in the skin of the guinea pig with concentrations of histamine as high as 1:100. Ramsdell[8] on the other hand, demonstrated reactions with a 1:1,000,000 dilution of histamine.

We have no explanation for the inability of Lamson and Pope and of Darsie, *et al.* to obtain positive reactions to histamine other than that suggested by Follensby and Hooker[9] that some strains of guinea pigs may be more reactive toward histamine than others.

Summary. The skin of old guinea pigs is more difficult to sensitize passively than the skin of young guinea pigs. This difference in response is not due to differences in the reactivity of old and of young guinea pigs toward histamine since both old and young guinea pigs react toward this substance to the same degree.

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7 Darsie, M. L., Perry, S. M., Rosenfeld, D., and Zaro, J. A., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 278.

8 Ramsdell, Susan G., *J. Immunol.*, 1928, **15**, 305.

9 Follansby, E. M., and Hooker, S. B., *J. Immunol.*, 1944, **49**, 353.

15987

Susceptibility of the English Sparrow (*Passer domesticus*) to Infection with Psittacosis Virus of Pigeon Origin.

Dorland J. Davis.   (Introduced by Charles Armstrong.)

From the Division of Infectious Diseases, National Institute of Health, Bethesda, Md.

The demonstration of ubiquitous infection among feral pigeons with psittacosis or psittacosis-like viruses[1,2,3] emphasizes the possibility of infection in the common English or house sparrow *(Passer domesticus)* because of its habit of feeding in association with pigeons. Various other members of the order Passeriformes have been shown to be naturally infected with psittacosis virus.[4]

Attention also was directed toward this possibility by an illness diagnosed serologically in this laboratory as psittacosis which was encountered in a woman physician who had handled a sick English sparrow. The onset of illness was on December 2, 1945 just 2 weeks after the patient had killed a sick and emaciated English sparrow which had frequented a bird feeding tray at her home. The patient also had scraped and cleaned the tray of droppings. A few pigeons belonging to a neighboring loft were noticed nearby occasionally. The illness was not severe and consisted of fever lasting for 6 days, (the highest temperature was 102.5°F) chills, severe sweats, and a dry cough. Abnormal physical findings were limited to the presence of rales over the lung bases. A roentgenogram of the chest taken 15 days after onset revealed an area of increased density at the left base and an increase in width of the hilar shadow. The specific diagnosis was not considered during the acute illness so that material for virus isolation studies was not available. Serum taken 29 days after onset fixed complement in the presence of both psittacosis

antigen and commercial *Lymphogranuloma venereum* antigen (Lygranum) in a dilution of 1:32. Serum taken 10 weeks after onset also fixed complement in the presence of psittacosis antigen but not in the presence of Q fever antigen. Samples of serum which had been collected for another purpose 3 and 5 months prior to the onset did not fix complement. While it was not possible to state definitely that the source of virus was the English sparrow, the question was considered worthy of further study.

Efforts to investigate the susceptibility of the English sparrow to psittacosis virus were undertaken in two ways. The first was to seek evidence of natural infection by attempts to recover the virus from birds captured in the vicinity of Washington, D. C. and by testing their serum for the presence of complement fixing antibodies. The second was to test directly the susceptibility of this species by the inoculation of captured birds with a known strain of virus.

Sparrows were captured in a wire funnel trap in 4 different locations in the Washington, D. C. area during 1946 and 1947. The spleen, liver, and a kidney of each bird to be tested for the presence of virus were ground together in a mortar, and a 10% suspension in 0.85% NaCl solution prepared. This was inoculated intraperitoneally into stock white mice (NIH strain). A week or ten days later the mice were autopsied and a normal saline suspension of the spleen inoculated intracranially into a second lot of mice. A third transfer was routinely made intracranially. Approximately half of the birds were first bled from the heart by passing a 22-gauge needle through the suprasternal notch in the midline and parallel to the vertebral column. Complement fixation tests of the serum were performed according to the technique devised

[1] Zichis, J., Shaughnessy, H. J., and Lembke, C., *J. Bact.*, 1946, **51**, 616.

[2] Labzoffsky, N. A., *Canad. J. Pub. Health*, 1947, **38**, 86.

[3] Davis, D. J., and Ewing, C. L., *Pub. Health Rep.*, 1947, **62**, 1484.

[4] Meyer, K. F., *Medicine*, 1942, **21**, 175.

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virus by inoculating mice intracranially with an approximate 10% suspension of the organs in 0.85%, NaCl solution. The presence of the characteristic clusters of elementary bodies in impression smears of the brain of mice dying 3 to 7 days after inoculation with the suspected material was taken as evidence of the presence of virus in the organ tested.

Birds inoculated intracranially died 3 to 15 days later and showed no gross abnormalities. Virus was present in the kidney and combined liver and spleen as well as in the brain.

In birds inoculated intraperitoneally the spleen was enlarged and soft, and the liver showed gross areas of focal necrosis, while the remaining organs appeared normal. Virus was recovered from the brain, kidney, and combined liver and spleen.

The oral-gastric route of infection was selected as approximating a natural mode of bird to bird transmission of virus. It was recovered from the organs of 7 of the 15 birds inoculated by this route which survived the first 7 days, and was present in the kidneys of 6 of these. Serum from 2 birds killed on the 14th day, from only one of which virus was recovered, fixed complement in the presence of psittacosis antigen, but serum from 2 others from which virus was recovered did not fix complement. Six other samples from uninfected birds were negative. The failure to demonstrate antibodies in birds from which virus is recovered may be due to a delay in antibody production as has been observed in pigeons.[6]

Summary. A serologically diagnosed case of psittacosis following exposure to a sick English sparrow and its droppings suggested an investigation of this species of bird as a carrier of psittacosis virus. 103 sparrows were examined for presence of active virus and serum from 59 of them were tested for complement fixing antibodies, but no evidence of natural infection was obtained. Four birds inoculated intracranially, 2 inoculated intraperitoneally and 7 of 15 inoculated by the oral-gastric route with psittacosis virus of pigeon origin became infected and virus was recovered from their organs.

6 Meyer, K. F., Eddie, B., and Yanamura, H. Y., PROC. SOC. EXP. BIOL. AND MED., 1942, 49, 609.

15988

Tyrosine Tolerance Test in Pregnancy and the Puerperium.*

ERNEST W. PAGE.

From the Division of Obstetrics and Gynecology, University of California Medical School, San Francisco.

We have shown previously that in normal pregnancy there is a marked increase in the excretion of both ingested and intravenously administered histidine, and that this is associated with a reduction of renal tubular reabsorption as well as with a delayed gastrointestinal absorption of this particular amino acid.[1,2] There is no apparent change, however, in the rate at which histidine disappears from the blood stream. The strange rejection of this important amino acid by both the renal and gastrointestinal epithelium during normal pregnancy is unexplained; we wished, therefore, to compare this finding with the metabolism and excretion of another amino acid, tyrosine, by the maternal organism. It would be desirable to know, furthermore, whether a tyrosine tolerance test might be of use as a measure of liver function in the toxemias of late pregnancy.

Following the oral ingestion of as much as

1 Page, E. W., West. J. Surg., 1943, 51, 482.

2 Page, E. W., Am. J. Obstet. and Gynec., 1946, 51, 553.

TABLE I.

Results of Inoculation by Different Routes of the English Sparrow with Psittacosis Virus of Pigeon Origin.

| No. of bird | Route of inoculation | Result in days after inoculation | Complement fixation titer of bird serum | Recovery of virus from bird organs by intracranial mouse inoculation | | |
				Brain	Spleen and liver combined	Kidney
965-1	intracranial	dead 3 days	—	+	0	0
965-2	,,	,, 3 ,,	—	+	.	0
965-3	,,	,, 9 ,,	—	+	+	0
965-4	,,	,, 15 ,,	—	+	+	+
990-1	intraperitoneal	,, 7 ,,	—	—	+	—
990-2	,,	,, 15 ,,	—	+	+	+
1047-3	oral-gastric	sacrificed 14 days	1/64	—	+	0
1047-4	,, ,,	,, 14 ,,	0	—	0	0
1057-1	,, ,,	dead 10 days	—	—	0	0
1057-2	,, ,,	sacrificed 14 days	0	—	+	+
1057-3	,, ,,	,, 14 ,,	1/256	—	0	0
1106-2	,, ,,	,, 14 ,,	0	—	0	0
1118-5	,, ,,	,, 14 ,,	0	—	0	0
1118-6	,, ,,	,, 14 ,,	0	—	0	0
1119-2	,, ,,	dead 9 days	—	—	0	0
1119-3	,, ,,	,, 11 ,,	—	. —	+	+
1119-4	,, ,,	,, 11 ,,	—	—	+	+
1119-5	,, ,,	sacrificed 11 days	0	—	+	+
1144-1	,, ,,	dead 8 days	—	—	+	+
1144-2	,, ,,	sacrificed 14 days	0	—	+	+
1144-3	,, ,,	,, 14 ,,	0	—	0	0

+ Virus recovered.
0 Virus not recovered.
— Not attempted.

by Bengtson[5] for rickettsial diseases. The antigen was prepared from psittacosis virus recovered from a parrot, grown in allantoic fluid of the developing chick embryo and killed with formalin or phenol.

Psittacosis or psittacosis-like virus was not isolated from any of the 103 sparrows examined. In two instances an unidentified bacillus apparently originating in the birds caused the death of the mice. No complement fixing psittacosis antibodies were detected in the 59 samples of serum tested.

Experimental Infection. In order to investigate the susceptibility of this species to infection with psittacosis virus sparrows were inoculated by 3 different routes with virus recently isolated from a feral pigeon. A total of 37 birds were inoculated, 4 intracranially under ether anaesthesia, 2 intraperitoneally, and 31 by dropping the inoculum into the throat or by passing a blunt needle down the esophagus of the anaesthetized bird. Due to trauma and the difficulty of keeping wild

5 Bengtson, Ida A., *Pub. Health Rep.*, 1944, 59, 402.

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White mice inoculated intracranially with each inoculum as a control died, and impression smears of the brain stained by Machiavello's stain showed typical clusters of elementary bodies in each instance.

Table I shows the results of inoculation of virus into the 21 birds which survived the trauma of capture and inoculation. Birds surviving until the 14th or 15th day after inoculation were bled and the serum tested for complement fixing antibodies. The sacrificed birds and those which died were autopsied, and tested for the presence of

virus by inoculating mice intracranially with an approximate 10% suspension of the organs in 0.85%, NaCl solution. The presence of the characteristic clusters of elementary bodies in impression smears of the brain of mice dying 3 to 7 days after inoculation with the suspected material was taken as evidence of the presence of virus in the organ tested.

Birds inoculated intracranially died 3 to 15 days later and showed no gross abnormalities. Virus was present in the kidney and combined liver and spleen as well as in the brain.

In birds inoculated intraperitoneally the spleen was enlarged and soft, and the liver showed gross areas of focal necrosis, while the remaining organs appeared normal. Virus was recovered from the brain, kidney, and combined liver and spleen.

The oral-gastric route of infection was selected as approximating a natural mode of bird to bird transmission of virus. It was recovered from the organs of 7 of the 15 birds inoculated by this route which survived the first 7 days, and was present in the kidneys of 6 of these. Serum from 2 birds killed on the 14th day, from only one of which virus was recovered, fixed complement in the presence of psittacosis antigen, but serum from 2 others from which virus was recovered did not fix complement. Six other samples from uninfected birds were negative. The failure to demonstrate antibodies in birds from which virus is recovered may be due to a delay in antibody production as has been observed in pigeons.[6]

Summary. A serologically diagnosed case of psittacosis following exposure to a sick English sparrow and its droppings suggested an investigation of this species of bird as a carrier of psittacosis virus. 103 sparrows were examined for presence of active virus and serum from 59 of them were tested for complement fixing antibodies, but no evidence of natural infection was obtained. Four birds inoculated intracranially, 2 inoculated intraperitoneally and 7 of 15 inoculated by the oral-gastric route with psittacosis virus of pigeon origin became infected and virus was recovered from their organs.

6 Meyer, K. F., Eddie, B., and Yanamura, H. Y., PROC. SOC. EXP. BIOL. AND MED., 1942, 49, 609.

15988

Tyrosine Tolerance Test in Pregnancy and the Puerperium.*

ERNEST W. PAGE.

From the Division of Obstetrics and Gynecology, University of California Medical School, San Francisco.

We have shown previously that in normal pregnancy there is a marked increase in the excretion of both ingested and intravenously administered histidine, and that this is associated with a reduction of renal tubular reabsorption as well as with a delayed gastrointestinal absorption of this particular amino acid.[1,2] There is no apparent change, however, in the rate at which histidine disappears from the blood stream. The strange rejection of this important amino acid by both the renal and gastrointestinal epithelium during normal pregnancy is unexplained; we wished, therefore, to compare this finding with the metabolism and excretion of another amino acid, tyrosine, by the maternal organism. It would be desirable to know, furthermore, whether a tyrosine tolerance test might be of use as a measure of liver function in the toxemias of late pregnancy.

Following the oral ingestion of as much as

1 Page, E. W., West. J. Surg., 1943, 51, 482.
2 Page, E. W., Am. J. Obstet. and Gynec., 1946, 51, 553.

TABLE I.

Results of Inoculation by Different Routes of the English Sparrow with Psittacosis Virus of Pigeon Origin.

No. of bird	Route of inoculation	Result in days after inoculation	Complement fixation titer of bird serum	Recovery of virus from bird organs by intracranial mouse inoculation		
				Brain	Spleen and liver combined	Kidney
965-1	intracranial	dead 3 days	—	+	0	0
965-2	,,	,, 3 ,,	—	+	+	0
965-3	,,	,, 9 ,,	—	+	+	+
965-4	,,	,, 15 ,,	—	+	+	+
990-1	intraperitoneal	,, 7 ,,	—	—	+	—
990-2	,,	,, 15 ,,	—	+	+	+
1047-3	oral-gastric	sacrificed 14 days	1/64	—	+	0
1047-4	,, ,,	,, 14 ,,	0	—	0	0
1057-1	,, ,,	dead 10 days	—	—	0	0
1057-2	,, ,,	sacrificed 14 days	0	—	+	+
1057-3	,, ,,	,, 14 ,,	1/256	—	0	0
1106-2	,, ,,	,, 14 ,,	0	—	0	0
1118-5	,, ,,	,, 14 ,,	0	—	0	0
1118-6	,, ,,	,, 14 ,,	0	—	0	0
1119-2	,, ,,	dead 9 days	—	—	0	0
1119-3	,, ,,	,, 11 ,,	—	—	0	0
1119-4	,, ,,	,, 11 ,,	—	—	+	+
1119-5	,, ,,	sacrificed 11 days	0	—	+	+
1144-1	,, ,,	dead 8 days	—	—	+	+
1144-2	,, ,,	sacrificed 14 days	0	—	+	+
1144-3	,, ,,	,, 14 ,,	0	—	0	0

+ Virus recovered.
0 Virus not recovered.
— Not attempted.

by Bengtson[5] for rickettsial diseases. The antigen was prepared from psittacosis virus recovered from a parrot, grown in allantoic fluid of the developing chick embryo and killed with formalin or phenol.

Psittacosis or psittacosis-like virus was not isolated from any of the 103 sparrows examined. In two instances an unidentified bacillus apparently originating in the birds caused the death of the mice. No complement fixing psittacosis antibodies were detected in the 59 samples of serum tested.

Experimental Infection. In order to investigate the susceptibility of this species to infection with psittacosis virus sparrows were inoculated by 3 different routes with virus recently isolated from a feral pigeon. A total of 37 birds were inoculated, 4 intracranially under ether anaesthesia, 2 intraperitoneally, and 31 by dropping the inoculum into the throat or by passing a blunt needle down the esophagus of the anaesthetized bird. Due to trauma and the difficulty of keeping wild

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FIG. 1.
Tyrosine tolerance curves on 8 normal pregnant women and 2 women with severe toxemia (indicated by x). All values in 10 normal non-pregnant women fell within shaded area.

10 g of tyrosine by normal individuals, there are no significant changes in the urinary output of tyrosyl compounds.[3] The shape and height of the blood curve observed after such ingestion is therefore dependent upon two factors: the rate of absorption from the gastro-intestinal tract and the rate of metabolism (utilization or destruction) of this compound. Bernhart and Schneider[4] followed the tyrosyl concentration of the blood at hourly intervals after the oral administration of 4 g of tyrosine, and suggested that this procedure be used as a test of liver function. The impaired metabolism of tyrosine associated with diseases of the liver results in a high initial level followed by an abnormal increase in the blood level at one hour and a delayed return to normal. In this respect, the type of curve obtained is analogous to the glucose tolerance curve associated with impaired carbohydrate metabolism. When there is delayed absorption from

the gastrointestinal tract, on the other hand, as noted in patients with untreated pernicious anemia, the peak is simply delayed until about three hours after ingestion but is not abnormally elevated.[5]

Methods. Eight volunteer subjects with normal pregnancies of 7 to 9 months' duration, one patient with eclampsia and one with a severe grade of preeclampsia were selected for study. Seven additional women with normal pregnancies were studied from 1 to 36 hours after delivery. Ten control subjects of similar age were selected from preoperative patients on the gynecologic service. All of the latter group were in good general health with no history of liver disease. Each woman was fasting on the morning of the test, and was given 4 g of tyrosine in casein solution. The methods of analysis employed, based upon the Millon reaction, were identical with those of Bernhart and Schneider,[4] except that blood samples were taken at intervals of 45 minutes

3 Medes, G., *Biochem. J.*, 1932, 26, 917.

4 Bernhart, F. W., and Schneider, R. W., *Am. J. Med. Sci.*, 1943, 205, 636.

5 Swenseid, M. E., and Bethell, F. H., *Proc. Centr. Soc. Clin. Res.*, 1944, 17, 40.

FIG. 2.
Tyrosine tolerance curves on 7 normal puerperal women. Figures in circle indicate the number of hours after delivery.

instead of hourly. This resulted in a change in the height and shape of the normal curve. In half the subjects of the normal non-pregnant and pregnant groups, urine samples were collected for a period of 8 hours before and 8 hours after administration of the tyrosine. These were analyzed for tyrosyl compounds by the Millon reaction as developed by Folin and Ciocalteu[6] and modified for urine determinations by Medes.[5]

Results. The fasting blood tyrosyl concentration of the 10 non-pregnant women was 1.44 mg/100 ml $\pm$ 0.09, with a standard deviation of 0.28. This compares favorably with the value of 1.4 as previously determined by the same method,[4] and with the value of 1.48 as determined by Hier and Bergheim[7] using microbiological methods. The fasting blood level of the 10 pregnant women was definitely lowered, being 0.99 mg/100 ml

[6] Folin, O., and Ciocalteu, V., *J. Biol. Chem.*, 1927, **73**, 627.

[7] Hier, S. W., and Bergheim, O., *J. Biol. Chem.*, 1946, **163**, 129.

$\pm$ 0.12 with S.D. of 0.37. The difference between these means is 3 times the standard error of the difference and may be considered significant. On the other hand, the postpartum values were even higher than the non-pregnant level.

The tyrosine tolerance curves for the 8 normal pregnant women and the two toxemia cases are illustrated in Fig 1. It may be seen that the values remained low, but that the rate of rise is similar to that of normal non-pregnant women, though tending perhaps to be somewhat flatter. There was no apparent delay in gastro-intestinal absorption as described for cases of pernicious anemia. The average urinary output of tyrosyl compounds before the test was 147 mg/12 hrs, and this increased to an average of only 190 mg/12 hrs after the test. There were occasional falls rather than rises, and there were no discernible differences between the pregnant subjects, with or without toxemia, and the normal non-pregnant women.

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Soon after delivery, however, there ap-

peared *to be a shift in the metabolism of tyrosine. The type of curves (Fig. 2) suggest impaired liver function, but it is more likely that the shift represents a reduced rate of utilization of this amino acid for protein synthesis in contrast to an increased utilization prior to delivery. This is in keeping with the well-known transition from a positive to a negative nitrogen balance after delivery. In the two subjects tested only 1 and 3 hours post-partum, the blood values were still rising at 90 minutes, suggesting a delayed absorption. This may represent a persistence of the known delay in both stomach emptying time and absorption of foodstuffs during active labor. The fact that pregnancy and the puerperium alter the tyrosine tolerance curves in opposite directions from the normal, and the failure to find further alterations in the 2 severe toxemia cases, would make it hazardous to use this procedure as a liver function test in pregnant* or puerperal women.

Conclusions. 1. The fasting blood tyrosyl level is significantly lowered during normal pregnancy, but not in the puerperium.

2. The reduced tyrosine tolerance curve during pregnancy suggests an increased rate of metabolism for this amino acid. There is an immediate reversal of this effect after delivery.

3. Unlike histidine, the renal tubular reabsorption of tyrosine appears to be as complete in pregnant as in non-pregnant subjects.

4. No alterations in tyrosine tolerance were noted in a case of preeclampsia nor in a case of eclampsia.

5. The observed shifts in tyrosine metabolism, possibly concerned with similar shifts in rates of protein synthesis, would probably invalidate the tyrosine tolerance test as a measure of liver function in pregnant and puerperal women.

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Further Studies on Renal Hyperlipemia.*

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Previous work[1,2,3] has shown that in dogs and rats unilateral and bilateral nephrectomy and the parenteral administration of nephrotoxic agents like mercury bichloride, uranium nitrate and potassium dichromate are followed by an increased blood lipid concentration. This effect of bilateral renal ablation has been confirmed by Winkler and associates[4] in dogs, has been recorded by these authors in monkeys, and by Nekludow[5] in cats. Hyperlipemia has also been found in rats made experimentally nephritic either by injection of antikidney serum[6] or by immunization with streptococcus—kidney antigen.[7] Hence, the kidneys of dogs, cats, rats and monkeys seem to function as part of a mechanism which influences blood lipid concentration.

Whether hyperlipemia also follows nephrectomy or renal injury in human beings has been investigated, and animal experiments have been carried out in an attempt

1 Heymann, W., *Science*, 1942, 96, 163.

2 Heymann, W., and Clark, E. C., *Am. J. Dis. Child.*, 1945, 70, 74.

3 Heymann, W., and Sekerak, B., PROC. SOC. EXP. BIOL. AND MED., 1945, 60, 276.

4 Winkler, A. W., Durlacher, S. H., Hoff, H. E., and Mann, E. B., *J. Exp. Med.*, 1943, 77, 473.

5 Nekludow, W. N., *Z. f. d. ges. exp. Med.*, 1925, 47, 70.

6 Farr, L. E., Smadel, J. E., and Holden, R. F., Jr., PROC. SOC. EXP. BIOL. AND MED., 1942, 51, 178

7 Cavelti, P. A., and Cavelti, E. S., *Arch. Path.*, 1945, 40, 163.

TABLE I.
Effect of Unilateral Nephrectomy on Serum Lipids in Man.

		Cholesterol		Total lipids	
		Lowest value	Highest value	Lowest value	Highest value
		Mg %		Mg %	
Before	Nephrectomy	151	190	400	540
After	,,	129	200	310	560
Before	,,	114	182	490	650
After	,,	114	191	490	800
Before	,,	191	238	800	930
After	,,	102	237	700	930

TABLE II.
Effect of Oral Administration of Mercury Bichloride on Serum Lipids in Man.

Dose in g	Cholesterol, mg %		Total lipids, mg %		Lecithin, mg %	
	Lowest value	Highest value	Lowest value	Highest value	Lowest value	Highest value
1.0	210	258				
1.0	85	160	440	720		
2.0	52	100	290	400	136	223
3.0	100	116	370	490	167	186
2.5	35	150	470	620	173	221
2.5	180	272	.			

to clarify the mechanism of the hyperlipemia.

Methods. Cholesterol analyses were made according to Schoenheimer and Sperry's method[8] which had been adapted to use with a photoelectric colorimeter.[9] The total lipids were analyzed according to the gravimetric method of Wilson and Hammer;[10] phospholipids were determined after extraction according to the technic of Bloor, with the method of Benedict and Theis[11] and were calculated as lecithin.

Effect of Unilateral Nephrectomy on Serum lipids in Man. Three patients with hydronephrosis, pyohydronephrosis and renal tuberculosis, respectively, were subjected to unilateral nephrectomy. During a period of 4 to 8 days before operation, determinations of total lipids and total and free serum cholesterol were carried out 2 to 4 times and daily for 2 to 3 weeks after the removal of one kidney. (Table I) No postoperative hyperlipemia was observed.

Effect of Mercury Bichloride Poisoning on Serum Lipids in Man. Total lipid, cholesterol and phospholipid determinations were carried out each day or every other day for 5 to 14 days on the sera of 6 patients who had taken mercury bichloride orally. Four of the 6 suffered severe kidney damage and died of uremia. Table II shows that all of the lipid values were normal or low. The observations thus failed to confirm the results of Wichert, Jakowlewa and Pospeloff[12] who found high cholesterol values in the blood of 2 patients poisoned with mercury bichloride.

Effect of Repeated Intravenous Infusions on the Hyperlipemia observed in Dogs After Unilateral Nephrectomy. Because the 3 nephrectomized patients had received, after their operations, an intravenous infusion of 1000 cc of Ringer's solution and 1000 cc of 5% glucose solution, we investigated the effect in dogs of such infusions upon the development of the post-nephrectomy hyperlipemia. Three dogs were unilaterally nephrectomized†

[8] Schoenheimer, R., and Sperry, W. M., *J. Biol. Chem.*, 1934, **106**, 745.

[9] Fitz, F., *J. Biol. Chem.*, 1935, **109**, 523.

[10] Wilson, W. R., and Hanner, J. P., *J. Biol. Chem.*, 1934, **106**, 323.

[11] Benedict, S. R., and Theis, R. C., *J. Biol. Chem.*, 1924, **61**, 63.

[12] Wichert, M., Jakowlewa, A., and Pospeloff, S., *Z. f. klin. Med.*, 1924, **101**, 173.

† We are greatly indebted to Dr. Harry Goldblatt of the Institute of Pathology, Western Reserve University, School of Medicine, for performing the operations on the dogs.

FIG. 1.
Effect of intravenous infusions of equal parts of Ringer's and 5% glucose solutions on serum lipids after unilateral nephrectomy in one of 3 dogs.
U. N., Unilateral nephrectomy.
Iv. Inf., Intravenous infusions.
Total Lipids, □——□
Lecithin, ▲——▲
Cholesterol, ●——●

and given intravenous infusions every day or two after operation. Per kg body weight, 20 to 50 cc of equal parts of Ringer's and 5% glucose solution, were infused as in the nephrectomized patients. The infusion was by continuous intravenous drip for 1½ to 2 hours between 9 and 11 a.m. Blood samples were obtained for analysis before the infusions were started. Comparison of the results (Fig. 1) with those previously obtained[1,2] showed that the intravenous administration of the Ringer's and the glucose did not abolish, but did limit the extent of the post nephrectomy hyperlipemia. The changes in cholesterol, lecithin, and total lipid values observed were irregular and less marked in the dogs that received fluids after operation.

Effect of Repeated Intravenous Infusions on Hyperlipemia Observed in Dogs after Parenteral Administration of Mercury Bichloride. The effect of daily infusions on blood

FIG. 2.
Effect of intravenous infusions of equal parts of Ringer's and 5% glucose solutions on hyperlipemia following injection of mercury bichloride in one of 3 dogs.
Iv. Inf., Intravenous infusions.
Total Lipids, □——□
Lecithin, ▲——▲
Cholesterol, ●——●

lipids was also studied in 3 dogs injected subcutaneously with 5 mg per kg body weight of mercury bichloride one day previous to the first infusion. Fig II shows that the infusions had little influence on the hyperlipemia development.

Effect of Repeated Oral Administration of Carbon Tetrachloride on Hyperlipemia Observed in Dogs after Subcutaneous Injection of Mercury Bichloride. In dogs and rats the oral administration of mercury bichloride had been previously shown[1,2] not to induce the hyperlipemia regularly observed after its parenteral administration. In both instances renal injury is produced. Inasmuch as mercury
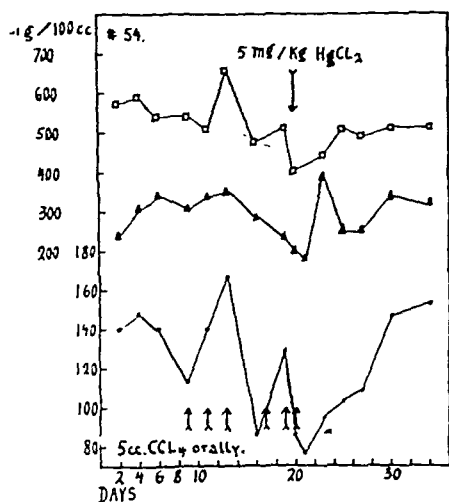
FIG. 3.
Effect on serum lipids in one of 3 dogs given carbon tetrachloride by stomach tube previous to a subcutaneous injection of mercury bichloride.
Total Lipids, □——□
Lecithin, ▲——▲
Cholesterol, ●——●

bichloride absorbed into the portal circulation might influence liver function, the effect of oral administration of carbon tetrachloride on the development of hyperlipemia was studied in dogs injected with mercury bichloride. After 5 cc of carbon tetrachloride had been given to 3 dogs by stomach tube at 2 to 4 day intervals for 12 to 18 days, 5 mg of mercury bichloride were injected subcutaneously. No elevation in blood lipid values was found in 2 of the dogs (Fig. 3). In the third, only a slight, delayed and irregular increase in total lipids and cholesterol was observed, and the phospholipids remained unchanged. The administration of carbon tetrachloride had thus inhibited the development of hyperlipemia otherwise observed after parenteral administration of mercury bichloride.

Discussion. The hyperlipemia observed in dogs after unilateral nephrectomy was not found in 3 human beings in whom one diseased kidney had to be removed. Although the conditions varied in the two series, it seems unlikely that the disparity is attributable to intravenous administration of fluids to the 3 nephrectomized patients after the operation. A possible explanation is that

in the dogs, healthy kidneys were removed, whereas the kidneys removed from the 3 patients were severely diseased. In 2 of the 3 patients, two-thirds of the removed kidneys seemed to be intact, but only 2 to 3mm of cortex was left in the 3rd kidney. Unimpaired anatomical structure of about two-thirds of renal tissue, however, does not imply functional integrity. Welsh, Mellin and Taylor[13] have shown that certain functional changes accompanying the renal hypertrophy which follows unilateral nephrectomy in human beings, are not observed when the removed kidney is severely diseased.

In dogs and rats the parenteral administration of mercury bichloride is regularly followed by hyperlipemia. This is not the case, however, when the mercury bichloride is given by stomach tube. The results of oral administration in human beings are in agreement with those obtained in animals given mercury bichloride by stomach tube. Why hyperlipemia develops only after parenteral and not after enteral administration of mercury bichloride, despite the fact that renal injury is produced in both instances, remains to be clarified. Suggestive evidence was obtained that liver function could interfere with the development of hyperlipemia. Liver injury, produced experimentally by carbon tetrachloride in 3 dogs, inhibited the development of the hyperlipemia otherwise regularly observed after the parenteral administration of mercury bichloride.

Summary. 1. Determinations of total lipids, total and free cholesterol were carried out in 3 patients subjected to unilateral nephrectomy. No hyperlipemia was observed.

2. Total lipids, cholesterol and phospholipids were determined in the serum of 6 patients with mercury bichloride poisoning. The values were normal or low.

3. Daily intravenous infusions of equal parts of 5% glucose and saline solution in dogs after unilateral nephrectomy and subcutaneous injection of mercury bichloride had some influence on the post nephrectomy hyperlipemia.

13 Walsh, G. A., Mellin, L., and Taylor, H. C., Jr., *J. Clin. Invest.*, 1944, **23**, 750.

FIG. 1.

Effect of intravenous infusions of equal parts of Ringer's and 5% glucose solutions on serum lipids after unilateral nephrectomy in one of 3 dogs.

U. N., Unilateral nephrectomy.
Iv. Inf., Intravenous infusions.

Total Lipids, □——□
Lecithin, ▲——▲
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Effect of intravenous infusions of equal parts of Ringer's and 5% glucose solutions on hyperlipemia following injection of mercury bichloride in one of 3 dogs.

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Total Lipids, □——□
Lecithin, ▲——▲
Cholesterol, ●——●

and given *intravenous infusions every day or two after operation.* Per kg body weight, 20 to 50 cc of equal parts of Ringer's and 5% glucose solution, were infused as in the nephrectomized patients. The infusion was by continuous intravenous drip for 1½ to 2 hours between 9 and 11 a.m. Blood samples were obtained for analysis before the infusions were started. Comparison of the results (Fig. 1) with those previously obtained[1,2] showed that the intravenous administration of the Ringer's and the glucose did not abolish, but did limit the extent of the post nephrectomy hyperlipemia. The changes in cholesterol, lecithin, and total lipid values observed were irregular and less marked in the dogs that received fluids after operation.

Effect of Repeated Intravenous Infusions on Hyperlipemia Observed in Dogs after Parenteral Administration of Mercury Bichloride. The effect of daily infusions on blood

lipids was also studied in 3 dogs injected subcutaneously with 5 mg per kg body weight of mercury bichloride one day previous to the first infusion. Fig II shows that the infusions had little influence on the hyperlipemia development.

Effect of Repeated Oral Administration of Carbon Tetrachloride on Hyperlipemia Observed in Dogs after Subcutaneous Injection of Mercury Bichloride. In dogs and rats the oral administration of mercury bichloride had been previously shown[1,2] not to induce the hyperlipemia regularly observed after its parenteral administration. In both instances renal injury is produced. Inasmuch as mercury

TABLE I.
Effect of Intravenous Injection of Insulin on Blood Glucose Concentration of Intact Cats.

The cats were fasted about 24 hr. Just after collection of a control blood sample from a femoral vein, the material indicated was injected. Additional blood samples were obtained 5, 10, 20, and 40 minutes after the injection. Glucose was estimated by the method of Nelson.[2]

Substance injected	Dose, ml or units per kg bodywt	Blood glucose, mg per 100 ml				
		Control	5 min.	10 min.	20 min.	40 min.
Water	0.1	84	86	78	80	85
	0.3	79	82	84	78	79
	1.0	91	97	94	94	92
0.156 M NaCl	0.1	83	82	87	82	87
	0.2	85	84	86	79	80
	1.0	90	92	87	91	90
Crystalline zinc insulin, Lilly	0.1	67	70	70	41	24
	2.0	54	72	86	51	30
,, ,, ,, Sharp & Dohme	0.1	70	81	76	64	55
	2.0	65	80	57	42	31
,, ,, ,, Squibb	0.1	72	82	78	59	42
	2.0	61	83	81	56	38
Amorphous insulin, Squibb	0.1	71	89	86	54	43
	2.0	67	93	83	42	32
Crystalline zinc insulin,* Lilly, pH 7	0.1	65	70	71	63	52
pH 7	2.0	81	94	89	76	47
pH 11	2.0	103	135	119	106	75
pH 3	2.0	71	112	101	83	62
NOVO insulin	0.1	75	70	64	57	40
	1.0	78	70	61	52	24
	2.0	80	78	64	48	28

* Solid material dissolved in 0.01 M HCl followed by sufficient 0.01 M NaOH to give pH indicated.

Transitory hyperglycemia was a common occurrence following administration of earlier preparations of amorphous insulin, but with the advent of crystalline insulin it appeared that the hyperglycemic effect was due to impurities in the amorphous material.[3,4] Dr. R. Levine, with whom the findings considered above were discussed, pointed out that Duve[5] recently stated that intravenous injection of one insulin preparation (Lilly) had a hyperglycemic effect and that another (NOVO) did not. Thus, test of the latter preparation was made in anesthetized and intact cats. As indicated by the data in the tables and other experiments, injection of NOVO insulin in doses up to 10 units per kg body weight was followed only by a decrease in glucose level.

Test for hyperglycemic response in species other than the cat was not made in the present work. However, it has been stated that in the dog constant intravenous injection of certain insulin preparations has a hyperglycemic effect,[6] and that in man the fall of blood glucose level begins more rapidly following intravenous injection of NOVO insulin than with other preparations.[7] Further, the glycogenolytic effect of some insulin

1 Olsen, N. S., and Klein, J. R., Fed. Proc., 1947, 6, 282.

2 Nelson, N., J. Biol. Chem., 1944, 153, 375.

3 Geiling, E. M. K., and DeLawder, A. M., J. Pharm. Exp. Therap., 1930, 39, 369.

4 Burger, M., and Kramer, H., Arch. exp. Path. Pharmakol., 1930, 156, 1.

5 Duve, C. de, Glucose, Insuline et Diabète, pp. XXVII, 307, Masson et Cie, Paris, 1945.

6 Levine, R., and Caren, R., Am. J. Physiol., in press.

7 McCulloch, W. S., unpublished data obtained in this laboratory.

4. Repeated oral administration of carbon tetrachloride prior to the subcutaneous injection of mercury bichloride in 3 dogs prevented the hyperlipemia in 2 of the animals.

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Hyperglycemia Induced by Certain Insulin Preparations.[*]

NORMAN S. OLSEN[†] AND J. RAYMOND KLEIN.

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In an attempt to demonstrate electroencephalographic changes characteristic of hypoglycemia, a preparation of insulin was injected intravenously into a paralyzed, artificially respired cat at 30-minute intervals, the initial dose being one unit per kg body weight and each subsequent dose twice that of the preceding one. Four hours after the initial dose, no change in the electroencephalogram was evident. In similar experiments a transitory increase in blood glucose concentration usually followed each injection of insulin and hypoglycemia did not develop.

Since the cats used in the experiments considered above were paralyzed with dihydro-β-erythroidine hydrobromide and the blood glucose levels were high, the effect of intravenous injection of insulin preparations on the concentration of blood glucose was tested in anesthetized (amytal) and intact cats. Representative data are given in Tables I and II. In addition to the experiments summarized in the tables, others were carried out in which different times of collection of blood were used, no injection was made, other preparations of amorphous insulin were used, arterial blood was assayed, doses of insulin intermediate to and greater than those indicated were employed, and in which insulin was given subcutaneously and intramuscularly. The effect of intravenous injection of insulin during hypoglycemia, induced by insulin, and hyperglycemia, induced by administration of glucose, was also tested.

The results of the experiments may be summarized as follows. Intravenous injection of water or salt solution in volumes comparable to the volumes of insulin used provoked no marked change, or certainly no consistent change, in blood glucose level. With intravenous injection of all preparations of insulin tested (except NOVO) the blood glucose concentration increased, the maximum occurring 5-10 minutes after injection. The glucose concentration returned to the control level 20-30 minutes after injection at which time hypoglycemia began to develop. The increase in glucose concentration was evident with 0.1 unit of insulin per kg body weight and marked with one unit. The hyperglycemic effect seemed to be somewhat more pronounced in the anesthetized than in the intact or paralyzed cat.

The effect was apparently independent of the blood glucose level, *e.g.* intravenous injection of 2 units of a preparation of crystalline zinc insulin per kg when, after intramuscular administration of insulin, the blood glucose level was gradually falling and had reached 18 mg per 100 ml, was followed by an increase in glucose level to 82 mg per 100 ml and disappearance of electroencephalographic changes characteristic of hypoglycemia; likewise, intravenous injection of insulin, at high blood glucose levels, induced by administration of glucose, was followed by increases in glucose level. With subcutaneous and intramuscular administration of the several preparations of insulin no increase in blood glucose level occurred.

TABLE I.

Effect of Intravenous Injection of Insulin on Blood Glucose Concentration of Intact Cats.
The cats were fasted about 24 hr. Just after collection of a control blood sample from a femoral vein, the material indicated was injected. Additional blood samples were obtained 5, 10, 20, and 40 minutes after the injection. Glucose was estimated by the method of Nelson.[2]

Substance injected	Dose, ml or units per kg bodywt	Blood glucose, mg per 100 ml				
		Control	5 min.	10 min.	20 min.	40 min.
Water	0.1	84	86	78	80	85
	0.3	79	82	84	78	79
	1.0	91	97	94	94	92
0.156 M NaCl	0.1	83	82	87	82	87
	0.2	85	84	86	79	80
	1.0	90	92	87	91	90
Crystalline zinc insulin, Lilly	0.1	67	70	70	41	24
	2.0	54	72	86	51	30
,, ,, ,, Sharp & Dohme	0.1	70	81	76	64	55
	2.0	65	80	57	42	31
,, ,, ,, Squibb	0.1	72	82	78	59	42
	2.0	61	83	81	56	38
Amorphous insulin, Squibb	0.1	71	89	86	54	43
	2.0	67	93	83	42	32
Crystalline zinc insulin,* Lilly, pH 7	0.1	65	70	71	63	52
pH 7	2.0	81	94	89	76	47
pH 11	2.0	103	135	119	106	75
pH 3	2.0	71	112	101	83	62
NOVO insulin	0.1	75	70	64	57	40
	1.0	78	70	61	52	24
	2.0	80	78	64	48	28

* Solid material dissolved in 0.01 M HCl followed by sufficient 0.01 M NaOH to give pH indicated.

Transitory hyperglycemia was a common occurrence following administration of earlier preparations of amorphous insulin, but with the advent of crystalline insulin it appeared that the hyperglycemic effect was due to impurities in the amorphous material.[3,4] Dr. R. Levine, with whom the findings considered above were discussed, pointed out that Duve[5] recently stated that intravenous injection of one insulin preparation (Lilly) had a hyperglycemic effect and that another (NOVO) did not. Thus, test of the latter preparation was made in anesthetized and intact cats. As indicated by the data in the tables and other experiments, injection of NOVO insulin in doses up to 10 units per kg body weight was followed only by a decrease in glucose level.

Test for hyperglycemic response in species other than the cat was not made in the present work. However, it has been stated that in the dog constant intravenous injection of certain insulin preparations has a hyperglycemic effect,[6] and that in man the fall of blood glucose level begins more rapidly following intravenous injection of NOVO insulin than with other preparations.[7] Further, the glycogenolytic effect of some insulin

[1] Olsen, N. S., and Klein, J. R., Fed. Proc., 1947, 6, 282.

[2] Nelson, N., J. Biol. Chem., 1944, 153, 375.

[3] Geiling, E. M. K., and DeLawder, A. M., J. Pharm. Exp. Therap., 1930, 39, 369.

[4] Burger, M., and Kramer, H., Arch. exp. Path. Pharmakol., 1930, 156, 1.

[5] Duve, C. de, Glucose, Insuline et Diabète, pp. XXVII, 307, Masson et Cie, Paris, 1945.

[6] Levine, R., and Caren, R., Am. J. Physiol., in press.

[7] McCulloch, W. S., unpublished data obtained in this laboratory.

TABLE II.

Effect of Intravenous Injection of Insulin on Blood Glucose Concentration of Anesthetized Cats. The cats were anesthetized by subcutaneous injection of 0.07 g sodium amytal per kg body weight. The other experimental details were the same as indicated in Table I.

Substance inj.	Dose, ml or units per kg body wt	Blood glucose, mg per 100 ml				
		Control	5 min.	10 min.	20 min.	40 min.
Water	0.1	53	51	56	57	66
	0.3	95	85	86	96	93
	1.0	89	95	95	80	83
0.156 M NaCl	0.1	58	62	55	58	45
	0.2	61	63	50	77	56
	1.0	60	58	62	59	60
Crystalline zinc insulin, Lilly	0.1	74	90	81	62	50
	2.0	82	138	137	86	48
,, ,, ,, Sharp & Dohme	0.1	61	78	62	50	37
	2.0	73	121	130	102	87
,, ,, ,, Squibb	0.1	63	91	86	51	39
	2.0	72	115	122	115	72
Amorphous insulin, Squibb	0.1	59	73	56	40	24
	0.2	63	101	71	59	42
Crystalline zinc insulin, Lilly, pH 7	0.1	71	84	79	66	52
pH 7	2.0	93	120	109	97	63
pH 11	2.0	78	104	84	88	52
pH 3	2.0	66	98	110	95	66
NOVO insulin	0.1	66	64	66	54	23
	1.0	70	65	48	35	19
	2.0	68	66	69	56	43

preparations on rat and rabbit liver *in vitro* is not obtained with NOVO insulin.[8]

The cause of the increase in blood glucose following intravenous injection of certain preparations of insulin is not known at present and for many purposes, *e.g.* the therapeutic, it is of no importance. However, in experiments concerned with the effect of these preparations of insulin on metabolism *in vitro* or in carbohydrate balance studies following intravenous injection of insulin, the hyperglycemic effect should obviously be considered.

Summary. Intravenous injection into cats of certain preparations of amorphous and crystalline insulin was found to provoke a transitory increase in blood glucose level. With one preparation, NOVO, no increase in glucose level occurred.

8 Sutherland, E. W., and Cori, C. F., *Fed. Proc.*, 1947, 6, 297. The observation cited was given in the amplified, verbal report.

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Physical-Chemical Factors in Agglutination of Sheep Erythrocytes by Influenza Virus.*

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The literature contains conflicting data concerning the agglutination of sheep erythrocytes by influenza virus. McLelland and Hare[1] failed to obtain agglutination with either of the A or B strains used in their tests; Burnet[2] and Clark and Nagler[3] obtained agglutination with some influenza virus strains but not with others.

The data included in the present paper show that agglutination of sheep erythrocytes by the WS strain[4] of influenza virus is dependent upon physical-chemical factors; when the requirements are satisfied, sheep cells yield essentially the same results as chicken cells in tests for the presence of influenza virus, and for the demonstration of influenza virus antibodies by the agglutination-inhibition test. The WS strain seemed particularly well suited to studies concerning the effects of physical-chemical factors on agglutination of sheep erythrocytes by influenza virus because this strain has been found by others not to cause agglutination of sheep cells under the usual test conditions.

Materials and Methods. The stock virus suspensions consisted of pooled allantoic fluid obtained from 30 to 40 eggs containing 13- to 14-days-old embryos, and which had been inoculated beneath the chorio-allantoic membrane 2 days previously, with an egg adapted strain. The normal allantoic fluid was pooled from eggs containing embryos of the same age as those from which the infected fluid was obtained. The erythrocyte suspensions were prepared in saline after thorough washing, from citrated sheep blood which contained 0.04% formalin for bacteriostatic purposes. McIlvaine's phosphate-citric acid buffer[5] was used in all tests; it seemed to be superior to phosphate buffers for the present purposes.

All tests for hemagglutination were made after the method described by Hirst,[6] in 10 x 75mm precipitin tubes. The total volume of ingredients was 0.6 ml. The degrees of agglutination were read on the basis of the cell patterns on the bottoms of the tubes, after the tests had been at room temperature sufficiently long (about 2 hours) for the erythrocytes to completely settle; they are recorded as no agglutination (0), partial agglutination (+), and complete agglutination (++).

Results. Table I shows the results of an experiment made to determine the effect of pH upon agglutination of sheep erythrocytes by different concentrations of WS-infected, and normal allantoic fluids. Test mixtures consisted of equal volumes (0.2ml) of allantoic fluid (infected or normal), buffer, and ¼% suspension of sheep erythrocytes.

In tests (Table I) with 1-100 dilution of infected allantoic fluid the virus caused agglutination of sheep erythrocytes over most of the pH range tested. However, in tests with other concentrations of infected allantoic fluid, agglutination depended upon pH, and to some extent upon the concentration of al-

1 McLelland, L., and Hare, R., *Canad. J. Pub. Health*, 1941, 32, 530.

2 Burnet, F. M., *Aust. J. Biol. and Med. Sc.*, 1942, 20, 81.

3 Clark, E., and Nagler, F. P. O., *Aust. J. Biol. and Med. Sc.*, 1943, 21, 103.

4 Smith, W., Andrewes, C. H., and Laidlaw, P. P., *Lancet*, 1933, 2, 66.

5 Clark, W. M., *The Determination of Hydrogen Ions*, Baltimore, Williams and Wilkins Co., 1928, p. 214.

6 Hirst, G. K., *Science*, 1941, 94, 22.

TABLE II.

Effect of Intravenous Injection of Insulin on Blood Glucose Concentration of Anesthetized Cats.
The cats were anesthetized by subcutaneous injection of 0.07 g sodium amytal per kg body weight.
The other experimental details were the same as indicated in Table I.

Substance inj.	Dose, ml or units per kg body wt	Blood glucose, mg per 100 ml				
		Control	5 min.	10 min.	20 min.	40 min.
Water	0.1	53	51	56	.57	66
	0.3	95	85	86	96	93
	1.0	89	95	95	80	83
0.156 M NaCl	0.1	58	62	55	58	45
	0.2	61	63	50	77	56
	1.0	60	58	62	59	60
Crystalline zinc insulin, Lilly	0.1	74	90	81	62	50
	2.0	82	138	137	86	48
,, ,, ,, Sharp & Dohme	0.1	61	78	62	50	37
	2.0	73	121	130	102	87
,, ,, ,, Squibb	0.1	63	91	86	51	39
	2.0	72	115	122	115	72
Amorphous insulin, Squibb	0.1	59	73	56	40	24
	0.2	63	101	71	59	42
Crystalline zinc insulin, Lilly, pH 7	0.1	71	84	79	66	52
pH 7	2.0	93	120	109	97	63
pH 11	2.0	78	104	84	88	52
pH 3	2.0	66	98	110	95	66
NOVO insulin	0.1	66	64	66	54	23
	1.0	70	65	48	35	19
	2.0	68	66	69	56	43

preparations on rat and rabbit liver *in vitro* is not obtained with NOVO insulin.[8]

The cause of the increase in blood glucose following intravenous injection of certain preparations of insulin is not known at present and for many purposes, *e.g.* the therapeutic, it is of no importance. However, in experiments concerned with the effect of these preparations of insulin on metabolism *in vitro* or in carbohydrate balance studies following intravenous injection of insulin, the hyperglycemic effect should obviously be considered.

Summary. Intravenous injection into cats of certain preparations of amorphous and crystalline insulin was found to provoke a transitory increase in blood glucose level. With one preparation, NOVO, no increase in glucose level occurred.

8 Sutherland, E. W., and Cori, C. F., *Fed. Proc.*, 1947, 6, 297. The observation cited was given in the amplified, verbal report.

TABLE II.
Comparison of Chicken with Sheep Erythrocytes for the Determination of Virus Content of Allantoic Fluid.

Erythrocyte suspension		2-fold dilution of fluid									
		1	2	3	4	5	6	7	8	9	10
Chicken	A	++	++	++	++	++	++	++	+	0	0
	B	0	0	0	0	0	0	0	0	0	0
Sheep	A	++	++	++	++	++	++	++	+	0	0
	B	0	0	0	0	0	0	0	0	0	0

A = WS infected allantoic fluid; B = Uninfected allantoic fluid.
The systems in the case of sheep cell tests contained pH 5.8 buffer, those in the chicken cell tests contained saline.

TABLE III.
Comparison of Chicken with Sheep Erythrocytes for the Determination of Influenza Virus Antibodies by the Agglutination-inhibition Test.

Erythrocyte suspension	Ferret serum	Initial 2-fold dilution of serum									
		3	4	5	6	7	8	9	10	11	12
Chicken	infl. A	0	0	0	0	0	0	0	++	++	++
	infl. B	0	+	++	++	++	++	++	++	++	++
Sheep	infl. A	++	+	0	0	0	0	+	++	++	++
	infl. B	++	++	++	++	++	++	++	++	++	++

Titration of Virus Suspension Used in Test.

Erythrocyte suspension	Initial 2-fold dilution						
	1	2	3	4	5	6	7
Chicken	++	++	++	+	0	0	0
Sheep	++	++	++	++	0	0	0

The systems in the case of the sheep cell tests contained pH 5.8 buffer, those in the chicken cell tests contained saline.

with strains of influenza B. The test mixtures in the case of the sheep cell tests contained equal volumes (0.2 ml) serum dilution, WS allantoic fluid diluted 1:32 with pH 5.8 buffer, and ¼% sheep erythrocytes. The tests with chicken cells differed in that the WS allantoic fluid was diluted 1:32 with saline, and the chicken cell suspension was ½%.

It is clear from the data (Table III) that the inhibitory effect of influenza A antiserums on agglutination of erythrocytes by the WS strain was detectable with the sheep erythrocytes as readily as with chicken cells. The inhibitory effect of the serum in the dilutions 2^{-4} to 2^{-9} in both instances must have been specific, because it was not obtained with influenza B virus antiserums. It is interesting that the non-specific effect of the serums (dilutions 2^{-3} and 2^{-1}) differed in the 2 tests.

In the case of tests with chicken cells, the serum caused inhibition of the virus hemagglutination. In the sheep cell tests, however, the serum itself agglutinated the erythrocytes.

Summary. The data indicate that agglutination of sheep erythrocytes by influenza virus (WS strain) is influenced by physical-chemical factors within the test systems, and especially by the hydrogen-ion concentration. Over a relatively wide pH range, agglutination of sheep cells is inhibited by substances present in allantoic fluid, normal and infected. The allantoic fluid inhibition is ineffective in the pH range 5.8 to 6.0. When the test systems are adjusted with pH 5.8 buffer, and ½% to ¼% cell suspensions are employed, sheep erythrocytes may be used with essentially the same results as obtained with chicken cells, for the determination of influ-

TABLE I.

Influence of pH upon Agglutination of Sheep Erythrocytes by Different Concentrations of Infected and of Uninfected Allantoic Fluids.

pH of test mixtures

Initial dilution of allantoic fluid		Saline	8.0	7.8	7.6	7.4	7.2	7.0	6.8	6.6	6.4	6.2	6.0	5.8	5.6	5.4	5.2	5.0	4.8	4.6
Undil.	A	+	+	+	+	+	o	o	+	+	+	+	+	+	+	+	+	P	P	H
	B		+	+	o	o	o	o	o	o	o	o	o	o	o	o	o	+	+	H
1-5	A	o	o	o	o	o	o	o	o	+	+	+	+	+	+	+	+	o	o	+
	B		o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	+	+	+
1-25	A	o	o	o	o	o	o	o	o	+	+	+	+	+	+	+	+	o	o	+
	B		o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	+
1-50	A	o	+	+	o	o	o	o	+	+	+	+	+	+	+	+	+	o	o	+
	B		o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	+	+
1-100	A	o	+	o	+	+	+	+	+	+	+	+	+	+	+	+	+	o	+	+
	B		o	o	o	+	+	o	o	o	o	o	o	o	o	o	o	o	+	+
1-200	A	o	o	o	+	+	+	+	+	+	+	+	+	+	+	+	o	o	+	+
	B		o	o	+	o	o	o	o	o	o	o	o	o	o	o	o	o	+	+
1-400	A	o	o	o	++	++	o	o	+	+	+	+	+	+	+	o	o	+	+	+
	B		o	o	+	++	+	o	o	o	o	o	o	o	o	o	o	+	+	+
No virus No allantoic fluid		o	o	o	+	++	+	o	o	o	o	o	o	o	o	o	o	++	+	++

0 = No agglutination; + = partial agglutination; ++ = complete aggl.; H = complete hemolysis; P = partial hemolysis; A = WS infected allantoic fluid; B = uninfected allantoic fluid.

lantoic fluid. Agglutination was caused by all dilutions of infected fluid, between pH 5.6 and pH 6.4; but in the more alkaline pH range the agglutination of sheep cells by virus was inhibited by substances present in the allantoic fluid, the degree of inhibition decreasing with decrease in concentration of allantoic fluid.

Buffer, in the absence of normal and infected allantoic fluids caused agglutination of the sheep cells between pH 4.6 and 5.0 and between pH 7.4 and 7.6; also, some agglutination was caused by concentrated normal and infected allantoic fluid at pH 7.8-8.0.

The combined effects of agglutination by virus, allantoic fluid and buffer (pH) on the one hand, and inhibition by allantoic fluid on the other, would render sheep cell tests unreliable over a large portion of the pH range tested. Nevertheless, at the pH range 5.8-6.0, sheep erythrocytes were so readily agglutinated by the virus that it seems likely that in a system adjusted to that pH, sheep erythrocytes should be satisfactory for influenza virus tests. It is to be noted, however, that concentration of cells is a very important factor. The best results are obtained with suspensions containing between ¼% and ½% of packed cells; very little agglutination is obtained with suspensions of 2% or more.

Table II compares sheep with chicken erythrocytes in tests to determine the virus content of allantoic fluid. The test mixtures contained equal volumes (0.2 ml). in the one instance of allantoic fluid (infected or uninfected), saline and ½% chicken cell suspension; and in the other instance, allantoic fluid, buffer pH 5.8, and ¼% sheep cell suspension.

It is clear (Table II) that the hemagglutinating effect of the infected allantoic fluid was approximately the same in tests with sheep cells as in tests with chicken cells.

Table III summarizes the results of an agglutination-inhibition test of known influenza virus antiserums versus the WS strain of virus. The pooled serums were from ferrets which had been repeatedly inoculated intranasally, in the one instance with strains of the virus of influenza A, and in the other instance

TABLE II.
Comparison of Chicken with Sheep Erythrocytes for the Determination of Virus Content of Allantoic Fluid.

Erythrocyte suspension		2-fold dilution of fluid									
		1	2	3	4	5	6	7	8	9	10
Chicken	A	++	++	++	++	++	++	++	+	0	0
	B	0	0	0	0	0	0	0	0	0	0
Sheep	A	++	++	++	++	++	++	++	+	0	0
	B	0	0	0	0	0	0	0	0	0	0

A = WS infected allantoic fluid; B = Uninfected allantoic fluid.
The systems in the case of sheep cell tests contained pH 5.8 buffer, those in the chicken cell tests contained saline.

TABLE III.
Comparison of Chicken with Sheep Erythrocytes for the Determination of Influenza Virus Antibodies by the Agglutination-inhibition Test.

Erythrocyte suspension	Ferret serum	Initial 2-fold dilution of serum									
		3	4	5	6	7	8	9	10	11	12
Chicken	infl. A	0	0	0	0	0	0	0	++	++	++
	infl. B	0	+	++	++	++	++	++	++	++	++
Sheep	infl. A	++	+	0	0	0	0	+	++	++	++
	infl. B	++	++	++	++	++	++	++	++	++	++

Titration of Virus Suspension Used in Test.

Erythrocyte suspension	Initial 2-fold dilution						
	1	2	3	4	5	6	7
Chicken	++	++	++	+	0	0	0
Sheep	++	++	++	++	0	0	0

The systems in the case of the sheep cell tests contained pH 5.8 buffer, those in the chicken cell tests contained saline.

with strains of influenza B. The test mixtures in the case of the sheep cell tests contained equal volumes (0.2 ml) serum dilution, WS allantoic fluid diluted 1:32 with pH 5.8 buffer, and ¼% sheep erythrocytes. The tests with chicken cells differed in that the WS allantoic fluid was diluted 1:32 with saline, and the chicken cell suspension was ½%.

It is clear from the data (Table III) that the inhibitory effect of influenza A antiserums on agglutination of erythrocytes by the WS strain was detectable with the sheep erythrocytes as readily as with chicken cells. The inhibitory effect of the serum in the dilutions 2^{-4} to 2^{-9} in both instances must have been specific, because it was not obtained with influenza B virus antiserums. It is interesting that the non-specific effect of the serums (dilutions 2^{-3} and 2^{-4}) differed in the 2 tests.

In the case of tests with chicken cells, the serum caused inhibition of the virus hemagglutination. In the sheep cell tests, however, the serum itself agglutinated the erythrocytes.

Summary. The data indicate that agglutination of sheep erythrocytes by influenza virus (WS strain) is influenced by physical-chemical factors within the test systems, and especially by the hydrogen-ion concentration. Over a relatively wide pH range, agglutination of sheep cells is inhibited by substances present in allantoic fluid, normal and infected. The allantoic fluid inhibition is ineffective in the pH range 5.8 to 6.0. When the test systems are adjusted with pH 5.8 buffer, and ½% to ¼% cell suspensions are employed, sheep erythrocytes may be used with essentially the same results as obtained with chicken cells, for the determination of influ-

TABLE I.

Influence of pH upon Agglutination of Sheep Erythrocytes by Different Concentrations of Infected and of Uninfected Allantoic Fluids.

Initial dilution of allantoic fluid		\ pH of test mixtures																		
		4.6	4.8	5.0	5.2	5.4	5.6	5.8	6.0	6.2	6.4	6.6	6.8	7.0	7.2	7.4	7.6	7.8	8.0	Saline
Undil.	A	+++ H	+	+ P	+	+	+	+	+	+	+	+	+	0	0	+	+	++	++	+
	B	+++ HH	+ P	+ P	+	+	+	+	+	+	+	+	+	0	0	0	0	+	+	0
1-5	A	+++	+	+	+	+	+	+	+	+	+	+	0	0	0	0	0	0	0	0
	B	+++	0	0	+	+	+	+	+	+	+	+	0	0	0	0	0	0	0	
1-25	A	+++	+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	0	+	0
	B	+++	0	0	0	0	0	0	0	+	+	0	0	0	0	0	0	0	0	
1-50	A	+++	0	+	+	+	+	+	+	+	+	+	+	+	+	0	+	0	+	0
	B	+++	0	0	+	+	+	+	+	+	+	+	0	0	0	0	0	0	+	
1-100	A	+++	++	0	0	0	0	0	0	0	+	0	0	+	+	+	+	0	0	0
	B	+++	+	0	0	0	0	0	0	0	+	0	0	0	+	+	0	0	0	
1-200	A	+++	++	0	0	0	+	+	+	+	+	+	+	0	+	++	++	0	+	0
	B	+++	+	0	0	0	0	0	0	0	0	0	0	0	0	+	0	0	0	
1-400	A	+++	++	+	0	0	0	0	0	0	+	0	0	0	+	++	+++	0	0	0
	B	+++	+	+	0	0	0	0	0	0	+	0	0	0	0	++	++	0	0	
No virus No allantoic fluid																				

0 = No agglutination; + = partial agglutination; ++ = partial aggl.; +++ = complete agglutination; H = partial hemolysis; HH = complete hemolysis.   A = WS infected allantoic fluid; B = uninfected allantoic fluid; P = partial.

lantoic fluid. Agglutination was caused by all dilutions of infected fluid, between pH 5.6 and pH 6.4; but in the more alkaline pH range the agglutination of sheep cells by virus was inhibited by substances present in the allantoic fluid, the degree of inhibition decreasing with decrease in concentration of allantoic fluid.

Buffer, in the absence of normal and infected allantoic fluids caused agglutination of the sheep cells between pH 4.6 and 5.0 and between pH 7.4 and 7.6; also, some agglutination was caused by concentrated normal and infected allantoic fluid at pH 7.8-8.0.

The combined effects of agglutination by virus, allantoic fluid and buffer (pH) on the one hand, and inhibition by allantoic fluid on the other, would render sheep cell tests unreliable over a large portion of the pH range tested. Nevertheless, at the pH range 5.8-6.0, sheep erythrocytes were so readily agglutinated by the virus that it seems likely that in a system adjusted to that pH, sheep erythrocytes should be satisfactory for influenza virus tests. It is to be noted, however, that concentration of cells is a very important factor. The best results are obtained with suspensions containing between ¼% and ½% of packed cells; very little agglutination is obtained with suspensions of 2% or more.

Table II compares sheep with chicken erythrocytes in tests to determine the virus content of allantoic fluid. The test mixtures contained equal volumes (0.2 ml), in the one instance of allantoic fluid (infected or uninfected), saline and ½% chicken cell suspension; and in the other instance, allantoic fluid, buffer pH 5.8, and ¼% sheep cell suspension.

It is clear (Table II) that the hemagglutinating effect of the infected allantoic fluid was approximately the same in tests with sheep cells as in tests with chicken cells.

Table III summarizes the results of an agglutination-inhibition test of known influenza virus antiserums versus the WS strain of virus. The pooled serums were from ferrets which had been repeatedly inoculated intranasally, in the one instance with strains of the virus of influenza A, and in the other instance

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H.M.	♂	31	Abortive	June 20	June 26	R-116	+	June 26	C-129	0
	♀	37	Indefinite	22	25	R-122	0	25	R-84	0*
	♂	8	Abortive	17	25	R-113	+	25	R-98	0
	♂	6	Paralytic	18	24	R-90	+	27	R-85	0*
	♂	2½	Abortive	17	25	R-117	+	27	R-96	0*
	♀	7 mo	„	15	25	R-115	+	27	R-101	0*
T.P.	♂	43	Indefinite	July 10	Aug. 3	R-119	0	Aug. 3	C-128	0*
	♂	41	None	—	4	R-125	0	3	R-103	0
	♀	11	Paralytic, mild	25	8	R-120	+	3	R-88	+
	♀	10	„ fatal	23	2	R-89	+	July 26	R-86	0*
	♂	4	None	—	2	R-112	+	Aug. 3	R-102	0
	♀	3	Abortive	28	4	R-91	+	3	R-87	0
C.D.	♂	28	None	—	Aug. 4	R-121	+	July 31	C-130	+
	♀	28	„	—	July 31	R-124	+	31	R-104	0
	♀	6	Paralytic	25	Aug. 4	R-109	+	26	R-99	++
	♀	2	Abortive	29	4	R-111	+	31	R-94	++
R.C.†	♂	36	Abortive	?Aug. 5	18	R-118	+	Aug. 19	C-132	+
	♀	34	None	—	18	R-123	0	19	C-131	xd3
	♂	7	Paralytic	Aug. 11	18	R-110	+	17	R-97	++
	♀	6	„	13	18	R-114	+	17	R-95	+

0* = susceptible on challenge to local heterologous strain isolated during same outbreak.

x = incompleted test.

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enza virus content of allantoic fluids, and for the demonstration of antibodies by the agglutination-inhibition test. The data are significant because of the bearing they have upon the mechanism of virus hemagglutina- tion.

15992 P

Widespread Distribution of Poliomyelitis in Households Attacked by the Disease.*

HERBERT A. WENNER AND WILLIAM A. TANNER.   (Introduced by C. J. Weber.)

From the Departments of Pediatrics and Bacteriology, and the Hixon Memorial Laboratory, University of Kansas Medical Center, Kansas City, Kansas.

Among patients admitted to the University of Kansas Hospitals because of poliomyelitis in 1946 it was clear that intercurrent illness had occurred in additional family members. Several households were selected for study on the basis that other illnesses had occurred at a time when poliomyelitis attacked a member of the group. Each member was sampled to determine whether poliomyelitis virus could be found in the oropharyngeal exudate and intestinal effluvia. Results obtained in a study of 4 households are reported here.

Material and methods. Throat Swabs: Cotton pledgets on applicator sticks were used to sample exudate from the oropharynx of each person. Pledgets moistened with exu- date were stored at -70°C in sterile individual screw-capped bottles containing 1 cc of distilled water. In the preparation of each specimen and its inoculation into monkeys we have used methods described in earlier papers.[1,2,3]

Stool Specimens: The manner of collection of stool samples has been described.[4] No more than 12 hours elapsed between collection of each specimen and storage in a dry ice chest.

The method of preparation and inoculation of monkeys with each specimen has also been described.[5,6] Each stool sample was inoculated into a monkey using the intraperitoneal and intranasal portals.

Monkeys: Rhesus *(Macaca mulatta)* and cynomolgus *(Macaca irus)* monkeys were used. A positive test in this study indicates that the monkey developed paralysis. In addition lesions consistent with those observed in poliomyelitis were observed in tissues from the spinal cord and elsewhere in the cerebral axis.

Results. The results of tests for poliomye- litis virus in oropharyngeal exudate and feces derived from each member in each of 4 house- holds appear in Table I.

Twenty people living in these 4 households were studied. Sixteen had poliomyelitis virus in their intestinal discharges, and among these 16, 7 had virus in the oropharynx. Of 12 children in these families, each had polio- myelitis virus in the stool specimen; 5 had virus in the oropharynx. Considering the 8 adults in these households, 4 had polio- myelitis virus in respective stool samples. Among these 4, virus was detected in the throat of 2 fathers. Poliomyelitis virus was not found in the remaining 4 parents.

1 Wenner, H. A., PROC. SOC. EXP. BIOL. AND MED., 1945, 60, 104.

2 Howe, H. A., Bodian, D., and Wenner, H. A., *Bull. Johns Hopkins Hosp.*, 1945, 75, 19.

3 Howe, H. A., and Bodian, D., *Neural Mechan- isms in Poliomyelitis,* Chapter III, 1942, The Com- monwealth Fund, New York.

4 Wenner, H. A., and Casey, A. E., *J. Clin. Invest.,* 1943, 22, 117.

5 Trask, J. D., Vignec, A. J., and Paul, J. R., PROC. SOC. EXP. BIOL. AND MED., 1938, 38, 147.

6 Howe, H. A., and Bodian, D., *Am. J. of Hyg.,* 1944, 40, 224.

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Type of illness	No. of cases	Mean interval in days between onset and sampling		Positive tests	
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Paralytic	6	8.6	5.3	6/6*	4/6
Abortive	7	8.3	8.3	7/7	2/7
Indefinite	2	14.0	14.0	0/2	0/2
None	5	—	—	3/5	1/4
	20			80%	37%

* Numerator—positive tests, denominator—individual tests completed.

Some facts concerning the members of these 4 households appear in Table II. The positive stool tests, aside from bringing persons who had poliomyelitis into view, confirm earlier studies.[7,8] Obviously it has been easy to detect poliomyelitis virus in stools. It is somewhat harder to find it in the oropharynx. If the detection of poliomyelitis virus in the throat has been irregular the results have a pattern. In other words it is not difficult to detect poliomyelitis in the oropharynx of a sick patient if sampling is done during the first few days of illness. It is harder to obtain specimens at an optimal period (1 to 5 days) in abortive and subclinical attacks of poliomyelitis unless symptoms occur at the same time or follow onset of illness in a recognized case in a household.

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Comment. It is quite clear that virtually all members in these households had poliomyelitis. As others[8,9] have, we found that adults as well as children had subclinical poliomyelitis. The detection of virus in 3 of 4 fathers is noteworthy, particularly in view of finding it in only one mother.

Our data are not extensive enough to determine how infection entered into the group. If the virus was introduced into the household by a carrier such an individual has been lost among other members who were found to harbor virus in the throat or stool. Indeed, there is suggestive evidence, based on the history of onset of illness, that infection occurred in these households as a result of exposure at a common source. Such an exposure must have been of short duration and taken place in a brief period of time.

Summary. A study of 4 households attacked by poliomyelitis provided evidence of widespread distribution of virus in the members of these units. There were 20 members in these households; 16 had poliomyelitis virus in their intestinal discharges; 7 had virus in the oropharynx.

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8 McClure, G. Y., and Langmuir, A. D., *Am. J. Hyg.,* 1942, **35,** 285.

9 Pearson, H. E., Brown, G. C., Rendtorff, R. C., Ridenour, G. M., and Francis, T., Jr., *Am. J. Hyg.,* 1945, **41,** 188.

15993

Effect of Hypothyroidism and Hyperthyroidism on Mammalian Skeletal Muscle.[*]

R. Diaz-Guerrero, J. D. Thomson, and H. M. Hines.

From the Department of Physiology, State University of Iowa, Iowa City.

Observations on clinical hyperthyroidism frequently mention as part of the syndrome asthenia, dystrophy and fine tremors of voluntary muscle. Remission of some of these symptoms often follows thyroidectomy. Pathological changes have been observed in such muscles.[1] Creatinuria is often present,[2] further indicating the existence of some muscular disorders. The present study reports the effect of hypo- and hyperthyroidism on the weight, strength and fatigability of skeletal muscle of the albino rat.

Methods. A total of 150 albino rats closely matched as to initial body weight, age and sex, was used in this study. One group of adult rats was made hypothyroid by including thiouracil in the diet[3] in amounts sufficient to lower the B.M.R. by approximately 20% (2 g thiouracil per kg powdered Purina Laboratory Chow). The hyperthyroid state was produced in one group by subcutaneous injection of 1.5 to 2.0 mg thyroxin twice a week. A third group served as untreated controls. Treatment was carried out for an average of 60 days before measurements were made of muscle weight and strength. The strength of the intact gastrocnemius-soleus muscles was determined by measuring the maximal isometric tension response of the muscles to slightly supermaximal condenser discharge stimuli applied to the tibial nerve with the animal under light ether anesthesia. The technics that were employed for strength measurements have been described in detail elsewhere.[4]

For the fatigue studies 35-day-old rats were divided into 3 groups. One group was given subcutaneous injections of thyroxin twice a week in amounts of 6 mg per kg body weight. A second group was reared on the thiouracil diet and a third group served as their untreated controls. Fatigue studies were made 90 days later on these animals while they were under light ether anesthesia. This was done by optically recording the isometric tension response of the gastrocnemius-soleus group to condenser discharge stimuli at slightly supermaximal intensity applied at the rate of 120 per second to the tibial nerve and induction shocks applied directly to the muscles at the rate of 120 per second. The records were analyzed for 2 indices of fatigue. The tensions maintained at 5 and 10 seconds after the onset of stimulation were calculated as per cent of the earlier maximal tensions and the area under the first 10 seconds of the tension curve was measured planimetrically from optical records.

Results. No significant difference was found (Table I) between the weight and strength of control and thiouracil treated animals. The thyroxin treated animals exhibited muscular weakness. This was shown by the findings of lower total tension, reduced tension per g of muscle. less tension per unit of body weight and a slightly lower ratio of muscle weight to body weight. Similar results were obtained in the studies on the younger group of animals which were under treatment for longer periods of time. Isometric tetanus tension elicited by nerve stimulation declined more rapidly in the hyperthyroid animals than in their controls. This was evidenced (Table II) by a reduction in the area of the tension curve and by a

[1] Alkanozy, M., *Deutsches Arch. f. Klin. Med.*, 1898, 61, 118.

[2] Palmer, W. W., Carson, D. A., and Sloan, L. W., *J. Clin. Invest.*, 1929, 6, 597.

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Summary. A study of 4 households attacked by poliomyelitis provided evidence of widespread distribution of virus in the members of these units. There were 20 members in these households; 16 had poliomyelitis virus in their intestinal discharges; 7 had virus in the oropharynx.

7 Paul, J. R., and Trask, J. D., *J. A. M. A.*, 1941, **116**, 493.

8 McClure, G. Y., and Langmuir, A. D., *Am. J. Hyg.*, 1942, **35**, 285.

9 Pearson, H. E., Brown, G. C., Rendtorff, R. C., Ridenour, G. M., and Francis, T., Jr., *Am. J. Hyg.*, 1945, **41**, 188.

15994

Further Observations on the Reproduction of Bacilli from Large Bodies in Proteus Cultures.*

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In a previous paper[1] the question was raised as to whether certain phenomena observed in Proteus may be sexual, in spite of the fact that fusion of cells is not involved. The experiments described in this note were undertaken to study this question. No evidence was obtained to prove that the influence of Proteus strains on each other is sexual, but the observations give further information concerning the remarkable antagonism between the strains.

The spreading filaments of Proteus when they meet filaments of another appropriate strain go through a peculiar transformation. They develop into large, round bodies, which continue to enlarge and reproduce either the usual small bacilli or tiny pleuropneumonia-like colonies. The influence of the spreading filaments on each other is quite specific. The usual small bacillary forms of Proteus are neither influenced by, nor exert any influence on, the filaments or small bacillary forms of another strain. The strains survive and retain their identity indefinitely in mixed cultures in broth. The motile filaments, so sensitive toward other Proteus strains, grow without hindrance through the various bacterial and mold colonies which they may encounter on the plates. The filaments are transformed into large bodies by relatively slight physical or chemical injuries: for example, refrigeration and exposure to tap water. The small bacillary forms are not sensitive to these influences.

This reaction of the filaments may be accidental. The spreading filaments develop only on a suitable medium and only have a short existence before they break again into small bacilli. It is unlikely that during the brief period of spreading they are exposed to adverse conditions which would change them into large bodies. Hence, the formation of large bodies at the contact zone of two spreading strains may be a natural function of the filaments.

Although the usual mechanism of sexuality, the fusion of cells, is absent in these processes, it seemed desirable to determine whether the essential purpose of sexuality, the crossing or segregation of properties, is not accomplished by some other unknown mechanism. To study this question large bodies were isolated with a micromanipulator from the contact line of two spreading cultures, and their descendants were compared with the parent strains. The technique used for the isolation of the large bodies was similar in principle to that described by Dickinson.† The large bodies were transferred to small pieces of transparent agar, and their development into bacterial colonies was observed under the microscope. This procedure assured that the colonies developing on the agar were the descendants of the large bodies. From single colonies of each culture so obtained, several strains were cultivated for further study.

These strains were studied with respect to their serological properties and their antagonism toward other Proteus strains. Cultures from different Proteus strains planted beside each other on a plate do not cover the agar entirely, but a sharp line of demarcation re-

[1] Dienes, L., Proc. Soc. Exp. Biol. and Med., 1947, 64, 165.

† See article of Stoughton.[2]

[2] *System of Bacteriology*, London, published by His Majesty's Stationery Office, 1931, 9, 105.

TABLE I.

Average Values, with Standard Errors, for Effect of Thyroxin and Thiouracil on Weight and Strength of Muscle.

	Control		Thyroxin		Thiouracil	
No. of animals	54		45		51	
Body wt before	173		171		170.	
" " after	269		240		248	
$\dfrac{\text{Muscle* wt}}{\text{Body wt}} \times 100$	0.650 ±	0.007	0.622 ±	0.007	0.676 ±	0.008
Total muscle* strength (g isometric tension)	3245	± 84	2599	± 149	3098	± 90
Tension per g muscle*	1825	± 35	1629	± 33	1806	± 33
$\dfrac{\text{Total muscle* tension}}{\text{Body wt}}$	11.87 ±	0.253	10.26 ±	0.268	12.12 ±	0.162

* Gastrocnemius-soleus.

TABLE II.

Average Values, with Standard Errors for Area Under Fatigue Curve, and Per Cent of Maximal Tension Remaining at 5 and 10 Seconds Through Direct and Indirect Stimulation.

Treatment	Area under 10 second fatigue curve muscle activated through		Nerve stimulation % max. tension		Muscle stimulation % max. tension	
	nerve cm²	muscle cm²	5 sec %	10 sec %	5 sec %	10 sec %
Thyroxin	50.7 ± 1.80	82.0 ± 2.24	27.9 ± 0.97	11.3	78.3	50.9
Control	75.4 ± 1.81	106.1 ± 2.27	44.6 ± 1.15	14.1	75.9	51.6
Thiouracil	63.3 ± 2.24	104.0	36.4 ± 1.54	11.8	76.8	53.8

lower per cent of tension existing after 5 seconds of stimulation. The thiouracil treated animals were inferior to their controls in respect to the maintenance of tension in muscle during motor nerve stimulation. No significant differences were noted between the three groups for the per cent of maximal tension decreases after 5 seconds of direct muscle stimulation. However, the tension area for direct muscle stimulation was significantly lower in the thyroxin treated than in the control animals.

Discussion. The evidence on the whole indicates that the hyperthyroid state is associated with some degree of muscular weakness and increased susceptibility to fatigue. The failure to maintain tetanus tension from nerve stimulation together with normal response to direct stimulation suggests that the factor of neuromuscular transmission may be involved. The hypothyroid condition resulting from thiouracil administration was accompanied by normal muscle weight and strength relationship except for a slightly more rapid decline in the tetanus tension elicited by motor nerve stimulation. It is to be pointed out that these studies deal only with the capacity of the muscles to develop and maintain tension in response to nerve stimulation and are not concerned with the stimulus pattern that is utilized in the natural activation of the muscles in reflex and voluntary activity. The asthenia that might result from subnormal activation would not be apparent in this type of study.

Summary. Albino rats were made hypothyroid by treatment with thiouracil and hyperthyroid by administration of thyroxin. After 60 days of treatment, gastrocnemius-soleus muscles of throxin treated animals showed weakness and increased susceptibility to fatigue through motor nerve stimulation. Thiouracil treatment for a like period of time resulted in no loss of muscle weight or strength, but did produce a slight increase in susceptibility to fatigue by indirect stimulation.

15995 P

Inhibition of Mumps Virus Multiplication by a Polysaccharide.

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Recently it was reported[1] that polysaccharides derived from various sources inhibit the multiplication of pneumonia virus of mice (PVM) in the mouse lung. The capsular polysaccharide of type B Friedländer bacillus proved to be the most effective of the preparations studied. Oxidation of the carbohydrate with periodic acid did not diminish its virus inhibiting activity, but the aldobionic acid derived by acid hydrolysis was without activity.

The effect of the Friedländer polysaccharide on the multiplication of viruses other than PVM has now been more extensively studied in the chick embryo. It has been found that the carbohydrate inhibits multiplication of mumps virus in the embryo, but .does not inhibit multiplication of influenza A, influenza B or Newcastle disease viruses. Moreover, the polysaccharide inhibits hemagglutination of chicken erythrocytes by mumps virus, and prevents adsorption of the virus to such cells.

Methods. The Friedländer polysaccharide (Fr.B,) used in these studies was prepared and purified as described previously.[2,3] The viruses employed were: influenza A, PR8 strain; influenza B, Lee strain; mumps; Newcastle disease. They were maintained by passage in the allantoic sac of chick embryos. Allantoic fluid was used as a source of virus for infectivity and hemagglutination experiments.

Experimental. As a routine, 10% chicken erythrocyte suspensions were treated with 5 mg per cc of polysaccharide for 3 hours at room temperature. Chicken erythrocytes adsorb the polysaccharide, and it is not removed
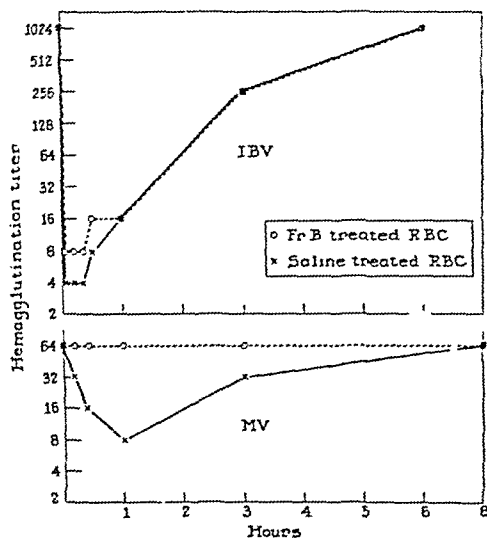
Fig. 1.
Comparison of the capacity of influenza B virus (IBV) and mumps virus (MV) to be adsorbed by and to be eluted from chicken red blood cells treated with Friedländer polysaccharide (Fr.B.).

by repeated washing. Such treated red blood cells are neither agglutinated by mumps virus nor are they capable of adsorbing the virus as is shown in Fig. 1. Treated cells are partially or completely inagglutinable by influenza A, influenza B and Newcastle disease viruses. However, these viruses are adsorbed by and eluted from treated erythrocytes just as they are with untreated red blood cells. The results of a representative experiment with influenza B virus are shown in Fig. 1. The polysaccharide has no demonstrable direct effect *in vitro* on any of the above viruses, including mumps.

When 0.5 to 1.0 mg of polysaccharide is injected into the allantoic sac of the chick embryo, a marked inhibition of the multiplication of mumps virus occurs even if the infecting dose is as great as 10^4 embryo infectious doses as is shown in Table I. Definite inhibition is obtained with as little as 50 μg of

[1] Horsfall, F. L., Jr., and McCarty, M., *J. Exp. Med.*, 1947, 85, 623.

[2] Heidelberger, M., Goebel, W. F., and Avery, O. T., *J. Exp. Med.*, 1925, 42, 701.

[3] Goebel, W. F., and Avery, O. T., *J. Exp. Med.*, 1927, 46, 601.

mains between the areas covered by different strains. Descendants of a single strain do not show this· phenomenon. Approximately 70 strains isolated from different sources were examined in this respect. All combinations of these strains which were tried were antago-nistic to each other. This antagonism between the strains offered a simple method to deter-mine whether all descendants of a large body are similar and correspond to one or the other of ·the original strains. If the cultures are thinly planted and begin to grow in isolated colonies, even a single colony with strain specificity different from the others is clearly· apparent in the plates.

Fifty-six large bodies were isolated from different combinations of 5 strains. Thirty-four of these developed into bacterial colonies and 18 did not grow. Their descendants were always similar to one of the original strains both in strain antagonism and serological properties. Neither segregation of properties nor crossing over from one strain to another was observed. However, it is of interest that with a single exception, in the combination of two strains, only large bodies belonging to one strain grew into bacillary colonies. .For example, 34 large bodies were isolated from the combination of strain No. 3 with other Proteus strains. Fourteen large bodies did not grow. The 20 which did develop never reproduced strain No. 3 but always the other strain. Large bodies produced in strain No. 3 by refrigeration are fully viable. Twelve large bodies were isolated from the mixture of strains Sm and No. 14, both of which dom-inated over strain No. 3. Six large bodies were viable all of which corresponded to strain

No. 14. In a combination of strains No. 14 and No. 52, 6 large bodies were isolated; 3 of these developed colonies corresponding to strain No. 52, one to strain No. 14 and 2 did not grow out. Strain No. 14 dominated over strain No. 3 and strain Sm, and was apparent-ly equal to or weaker than strain No. 52.

The dominance of one strain over the other is not genetic, but it is probably the result of the strain antagonism already discussed. When the strains come in contact, both respond by transformation into large bodies, but apparent-ly only the large bodies of one strain survive. The numerical relationship observed between the viable and non-viable large bodies may be influenced by the fact that large bodies are not produced in equal numbers by the differ-ent strains. Also, some are destroyed by the manipulation necessary for their isolation.

The observations in their present status give no information as to the function of the large bodies or the reproductive processes connected with them in Proteus. It is possible that their real significance is in connection with pleuropneumonia-like colonies and not with the direct reproduction of bacilli. The antagonism which appears in certain phases of growth between Proteus strains has thus far not been observed in other species.

Summary. Single large bodies were isolated from the contact line of 2 spreading Proteus strains. Their descendants were similar to one of the strains with respect to the prop-erties studied, and crossing or segregation of properties was not observed. With a single exception, only one strain was recovered from the large bodies formed at the contact of 2 strains.

15996 P

An Improved Benzene Extracted Complement Fixing Antigen for Certain Neurotropic Viruses.*

Carlos Espana[†] and W. McD. Hammon.

From the George Williams Hooper Foundation for Medical Research, University of California, San Francisco, Calif.

DeBoer and Cox[1,2] have recently described benzene-extracted mouse-brain and chick embryo complement fixing antigens for the diagnosis of neurotropic virus infections. The advantage claimed is that these antigens do not react with syphilitic sera that have been inactivated at only 60°C. We have now prepared antigens according to this new technic for Western and Eastern equine, St. Louis, Japanese B and Hammon-Reeves California viruses. Procedures were repeated several times for most viruses until several lots had been prepared. However, although the antigens completely fulfilled the criteria claimed by the authors, and represent an important advance, their sensitivity was slightly less than that of those we were accustomed to using (prepared by either the technic of Casals[3] or by a modification of that of Havens et al.,[4] centrifuging 10 per cent mouse-brain at 16,000 r.p.m.). Furthermore, the preparation described was prolonged (requiring about 4 days), and in our opinion unnecessarily dangerous (transferring and pulverizing powdered antigen from filter paper). We therefore undertook several experimental modifications of this technic. A more complete paper with extensive protocols is being published separately.

Preparation of Antigens. Mice are inoculated intracerebrally with a 10⁻² or 10⁻³ dilution of virus. The mice, when moribund, are anaesthetized, bled to death and the brains removed. The brains are then ground in a Waring blendor in pyrogen-free, fractionally distilled water to make a 20% suspension. After storage for 3 to 4 hours at 5°C, 25 ml amounts are rapidly shell frozen in 250 ml Pyrex bottles and then lyophilized. The dried tissue is then extracted with benzene for 1 hour at room temperature by adding a volume of benzene equivalent to twice the original aqueous suspension. The benzene is rapidly removed by filtration through a Gooch crucible filter under high vacuum. Two further benzene extractions are performed in the same filter, allowing 30 minutes at room temperature each time before applying vacuum. The extracted tissue is then transferred under a hood by inverting the Gooch filter over a wide mouth stemless funnel placed in the neck of a 250 ml Pyrex bottle and then tapping the crucible filter gently till the dried powder falls free. The remaining solvent is removed by applying negative pressure to the bottle. Saline is next added in the amount of the original volume and rehydration permitted to occur overnight at 5°C, then after centrifugation for 30 minutes at 10,000 r.p.m. the supernatant is removed and Merthiolate is added to a final dilution of 1:10,000. The antigen is ready for immediate use, but for prolonged storage or shipment it is again lyophilized. The antigen is complete within 36 hours after killing the mice.

Characteristics of the Antigen. All tests have been made using essentially the technic described by Casals.[3] The liquid antigen,

1 DeBoer, C. J., and Cox, H. R., J. Bact., 1946, 51, 613.

2 Ibid., J. Immunol., 1947, 55, 193.

3 Casals, J., J. Exp. Med., 1944, 79, 341.

4 Havens, W. P., Jr., Watson. D. W., Green, R. H., Lavin, G. I., and Smadel, J. E., J. Exp. Med., 1943, 77, 139.

TABLE I.

Effect of Friedländer Polysaccharide on the Multiplication of Mumps Virus in the Chick Embryo.

1st injection intra-allantoic 0.1 cc	Time between inj. hrs	2nd inj. intra-allantoic 0.1 cc	Mean hemagglutination titer‡ of allantoic fluids of embryos	
			Inj. with Fr.B. polysaccharide	Controls inj. with saline
Fr. B. polysaccharide*	3	Mumps virus 10^1 E.I.D.†	4	175
	3	10^2	1	264
	3	10^3	3	128
	3	10^4	4	245
Mumps virus 10^2 E.I.D.	3	Fr.B. polysaccharide	5	154
	24		2	154
	48		11	129
	72		240	—
	96		72	576
	120		384	—

* 1.0 mg per chick embryo.
† E.I.D. = chick embryo infectious doses.
‡ Mean of the reciprocals of the hemagglutination titers.

polysaccharide per embryo. In these experiments the concentration of virus in allantoic fluids was measured by the hemagglutination technique[4] 6 days following inoculation of the virus. As indicated in Table I polysaccharide may be injected 3 hours before, or as long as 48 hours after inoculation of mumps virus and still cause inhibition of multiplication of the virus. Multiplication of the virus in the allantoic sac is also inhibited when 5 mg of Fr. B. is injected into the embryonic yolk sac either 3 hours before or 3 hours after the inoculation of virus.

Comment. The role which the capsular polysaccharide of type B Friedländer bacillus plays in the inhibition of virus multiplication

is not yet understood. Nor is it yet possible to correlate the activity of this and other carbohydrates with similarities in chemical constitution. It is possible, however, that knowledge of the biochemical mechanism involved in this phenomenon may lead to an understanding of the intracellular systems concerned in the multiplication of viruses. The fact remains that a substance belonging to a well-defined class of chemical compounds has been found which exerts specific inhibition on the multiplication of certain viruses in the living host.

Summary. The capsular polysaccharide of type B. Friedländer bacillus inhibits the multiplication of mumps virus in the chick embryo and prevents adsorption of the virus by treated erythrocytes.

[4] Levens, J. H., and Enders, J. F., *Science*, 1945, **102**, 117.

15996 P

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prior to the final lyophilization has remained unchanged in all its characteristics when stored at 5°C for at least 6 months. The final, dry product goes into complete solution rapidly and requires no centrifugation before use. Its titer is the same as before lyophilization, and if any particular lot of antigen has a low titer, by adding only half the original volume of water to the powder the titer can be doubled. The reconstituted antigens have been tested after 3 months storage in the liquid state and have shown no decrease in titer or tendency to become anticomplementary.

These antigens when first prepared have infective titers of 10^{-3} or 10^{-4}. After a few weeks in the liquid state at 5°C they become practically nonvirulent.

No human serum, syphilitic or otherwise, among approximately 500 that we have tested has given a non-specific response after inactivation for 20 minutes at 60° C.

These antigens should be diluted further for routine use, for when concentrated, relatively low serum titers are obtained. The optimal dilution is determined by performing a combined, serial, two-fold immune guinea pig serum, and two-fold antigen titration. The highest dilution of antigen giving the highest serum titer is selected for use. This is usually a dilution of 1:4 to 1:16 of the antigen (1.25 to 5% brain) and represents about 8 to 16 antigen units. With guinea pig immune sera, antigens titer (in serial 2-fold antigen dilutions) from 1:64 or 1:128 (Western equine and California virus) to 1:256 or 1:512 (Japanese B, St. Louis and Eastern equine). At the optimal dilutions no overlapping can be demonstrated between any of our antigens and any heterologous hyperimmune sera, not even between Japanese B and St. Louis, although when an excess of antigen is used such overlapping is observed.

Human convalescent sera for Western equine infections have titered as high as 1:64 (original serum dilution) and for St. Louis and Japanese B (sera collected by W. M. H. from 1945 Okinawa epidemic) up to 1:512. Human convalescent sera give higher titers with these antigens than with any others we have ever prepared and there has been greater uniformity of titer between repeated preparations with the same virus. Moreover, these are the only antigens that have not given us occasional non-specific reactions with sera from febrile patients with other infections.

These antigens all show a tendency to protect complement during overnight incubation at 5°C and thus avoid certain difficulties shown by some other antigens when used with sera that are very slightly anticomplementary, though not detectably so in the anticomplementary control.

Summary. A time saving and safer procedure for preparing benzene extracted brain antigens is described. The resultant antigen is more sensitive and more specific than others previously tested by us. In its final form it is a stable, lyophilized product that can be easily shipped and can be used without centrifugation after rehydration.

15997 P

Excretion of Poliomyelitis Virus.*

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During the early stages of human poliomyelitis virus appears in the pharyngeal secretions and in the stools.[1,2,3,4] The experiments of Melnick[5] suggest that this phenomenon is excretory, since it occurs in the stools after parenteral inoculation, but little is known of the mechanism or pathways of excretion. The present study is part of an exploration into the mode of virus excretion.

Following an hypothesis proposed in another paper[6] and using monkeys, we have applied 20% virus (our *Cam* strain, 2nd-7th passages) for 10 minutes to the central end of the divided infraorbital branch of the right trigeminal nerve in the cheek ("nerve dip"), sedulously avoiding virus contamination of the skin and mucous membranes. Preliminary tests with rhesus (*M. mulatta*) showed that on the third day heavy lesions were usually present in the homolateral but not the contralateral semilunar (Gasserian) ganglion, while no more than minimal lesions were found, and then only occasionally, in the central components of the trigeminal system in the pons and medulla. At the same period, virus was detected in the homolateral but not in the contralateral ganglion. Virus was also found in pooled nasopharyngeal washings collected from the 2nd to the 5th day, inclusive.

With these preliminary data at hand, 4 cynomolgus monkeys (*M. irus*) of Philippine origin were similarly treated and materials collected and pooled serially on the second, third and fourth days after exposure, the animals being sacrificed on the fourth day. Nasopharyngeal washings were obtained by washing 0.85% NaCl solution through one nostril with a catheter and collecting the returning fluid from the other. This material as well as the stools and intestinal contents was concentrated by the technique described by one of us (R.J.S.)[7] and subinoculated intrathalamically into rhesus monkeys. The results are shown in Table I.

Discussion. In a previous paper[6] we have suggested on anatomical grounds the hypothesis that when poliomyelitis virus, which is known to be axonally conducted, has penetrated a body surface, entered the telodendria of a peripheral afferent neuron and ascended to the corresponding peripheral ganglia where it can multiply and infect adjoining neurons, it would then be in a position to spread not only centripetally to the CNS but also centrifugally back to the surfaces supplied by the same nerve system. This possibility arises because the peripheral neurons of most afferent nerves have T-shaped axons, one branch of which ascends to the CNS and the other is directed to the periphery. In the case of the trigeminal nerve, which was investigated in the present experiments, virus would thus be brought back by centrifugal spread to the terminal arborizations in the naso-oropharyngeal mucosa, which are stated to end free on the surface,[8] whence it might be

1 Taylor, E., and Amoss, H. L., *J. Exp. Med.*, 1917, 26, 745.

2 Paul, J. R., Salinger, R., and Trask, J. D., *J. A. M. A.*, 1932, 98, 2262.

3 Howe, H. A., Bodian, D., and Wenner, H. A., *Bull. Johns Hopkins Hosp.*, 1945, 76, 19.

4 Horstmann, D. M., Melnick, J. L., and Wenner, H. A., *J. Clin. Invest.*, 1946, 25, 270.

5 Melnick, J. L., *J. Immunol.*, 1946, 53, 277.

6 Faber, H. K., and Silverberg, R. J., *J. Exp. Med.*, 1946, 83, 329.

7 Silverberg, R. J., *Science*, 1945, 102, 380.

8 Schaeffer, J. P., *The Nose, Paranasal Sinuses, Nasolacrimal Passageways, and Olfactory Organ in Man*, P. Blakiston's Sons and Co., Philadelphia, 1920, p. 285.

TABLE I.

Distribution of Poliomyelitis Virus After Right Infraorbital Nerve Dip. Four Cynomolgus Monkeys. Cam Strain of Virus.

Days after inoculation	Nasopharyngeal washings	Stools	Semilunar ganglia		Washed pharyngeal wall
			Right	Left	
2	0	0	—	—	—
3	+*	0	—	—	—
4	+	+	+	0	0

* Non-paralytic case: cord histologically positive.
— Not tested.
+ Clinically positive: cord sections positive.
0 Cord sections negative; symptoms absent or very questionable.

excreted and detected in the overlying mucus.

Our experiments tentatively confirm the hypothesis in the following respects: (1) ascent by centripetal spread to the Gasserian ganglion; (2) limitation of infection to the nerve system in question during the first 3 days; (3) excretion of virus in the appropriate area on the third day. Avoidance of primary exposure of the mucosa to virus rules out implantation of virus on the mucosal surface. Excretion by the lymphatics is highly improbable, since lymph flow is away from rather than toward the surface.[9]

The very early appearance of virus on the nasopharyngeal mucosae is noteworthy and interesting in reference to similar findings in the human disease,[1-3] as is the fact that it

[9] Yoffey, J. M., and Drinker, C. K., *J. Exp. Med.*, 1938, **68**, 629.

appeared a day later in the stools than in the nasopharyngeal washings, which might be explained on the basis of swallowing.

This work is to be repeated and expanded.

Summary. Poliomyelitis virus when applied to the central end of a divided branch of the trigeminal nerve in the cheek travels centripetally to the corresponding semilunar ganglion within three days. Centrifugal spread to the nasopharyngeal surfaces, which are supplied by the trigeminal nerve, was demonstrated by detection of virus in the nasopharyngeal washings on the third and fourth days. Virus was also found in the stools on the fourth but not on the third day, suggesting that it had been swallowed. It is suspected that excretion, like invasion, of poliomyelitis virus occurs through axonal channels.

15998

Prolonged Survival of Adrenalectomized-Nephrectomized Rats on a Low Potassium Diet.*

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Adrenalectomized animals are extraordinarily sensitive to all types of stress, including

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the metabolic derangements following nephrectomy. During the course of investigations on the relation of the adrenal cortex to urea synthesis, considerable difficulty was met in maintaining life in adrenalectomized-nephrectomized animals long enough for satisfactory study. The limited survival was not influenced by the use of liberal amounts

of desoxycorticosterone acetate and saline for as long as seven days preoperatively. Durlacher and Darrow[1] have already shown that the survival of nephrectomized but otherwise normal rats may be significantly prolonged by preoperative potassium depletion by a low potassium diet. Since the adrenalectomized rat is particularly sensitive to potassium accumulation, such as occurs after nephrectomy, the effects of a low potassium diet on the survival of adrenalectomized-nephrectomized rats were investigated. It was found that not only was the survival time prolonged over that of adrenalectomized-nephrectomized rats on the chow diet, but it was also significantly extended beyond that of nephrectomized, but otherwise normal rats also on a low potassium diet.

Methods. Male rats of the Sprague-Dawley strain, weighing 170-200 g, were fed Rockland rat chow until the beginning of the experiment. For 3 days before operation the animals received the following diet: Casein (Technical), 180; sucrose, 570; peanut oil, 220; cod liver oil, 10; brewers' yeast, 20; bone ash, 20; sodium chloride, 5. This diet contains 1.4 mEq of potassium per 100 g,[‡] as compared to 24.6 mEq per 100 g of Rockland rat chow. The animals were allowed free choice of water and 1% saline in their drinking fountains throughout the experiment. On the evening of the third day, all food was removed, and the animals were anesthetized by the intraperitoneal injection of 3 mg nembutal per 100 g of body weight. After operation, the animals were bled 2 to 3 times daily from the tail, 0.3 ml of blood being removed and used for the determination of urea nitrogen by the xanthydrol method of Engel and Engel.[2] Total urea synthesis, expressed as milligrams of nitrogen per 100 g body weight per hour was calculated from the rise of blood urea nitrogen, assuming the urea to be equally distributed throughout the body

water, *i.e.* 75% of the body weight. Survival times were judged to be the last time the rats were known to be alive. Thus rats dying during the night were considered to have a shorter survival time than was actually the case. This error equally affected both adrenalectomized and control animals. The largest error introduced in this assumption was not more than 6 hours. Urea levels at the time of death were calculated by assuming no change in the rate of urea formation from the time of the last determination to the time of death. In several instances where samples were obtained within an hour or less of the time of death, this assumption was found to be valid.

Nephrectomies and adrenalectomies were performed simultaneously through bilateral subcostal incisions. In several animals, the adrenal area was examined histologically after death and the absence of adrenal rests demonstrated.

Results. Table I compares the survival time of nephrectomized-adrenalectomized rats on the chow diet and previously treated with 5 mg of desoxycorticosterone acetate for 3 days with those on the low potassium diet, and with nephrectomized rats with intact adrenals on the low potassium diet. It is apparent that preparation with the special diet prolonged the life of the adrenalectomized-nephrectomized rats to a significant degree ($p < 0.01$).[§] There was also a significant increase in the survival time of adrenalectomized rats as compared to those which had merely been nephrectomized ($p < 0.02$). When Upjohn's aqueous adrenal cortical extract[‖] was administered to the adrenalectomized rats at a dose level of 1 ml twice daily through the course of the experiment, the survival time was intermediate between the control and the adrenalectomized group, but was not significantly different from either group ($p > 0.05$).

1 Durlacher, S. H., and Darrow, D. C., *Am. J. Physiol.*, 1942, **138**, 577.

‡ The potassium analysis was performed by the Department of Biochemistry, Emory University.

2 Engel, M. G., and Engel, F. L., *J. Biol. Chem.*, 1947, **167**, 535.

TABLE I.
Survival Times and Terminal Blood Urea Nitrogen Levels After Nephrectomy.

	No. of rats	Survival time, hr	Terminal blood urea nitrogen, mg/ 100 ml
Adrenalectomized-nephrectomized, chow diet*	3	13.5 ± 2.3†	—
Adrenalectomized-nephrectomized, low potassium diet	7	98.4 ± 5.2	240 ± 5.1†
Nephrectomized, low potassium diet	7	77.1 ± 5.5	329 ± 35.92
Adrenalectomized-nephrectomized, low potassium diet, adrenal cortical extract	7	89.1 ± 3.4	264 ± 17.4

* Treated with 5 mg desoxycorticosterone acetate for 3 days prior to nephrectomy.
† Standard error of the mean.

The relation of the survival time to the degree of uremia is also shown in Table I which records the blood urea nitrogen concentration of the rats at the time of death. It was found that the final urea concentration of the adrenalectomized animals was lower than that of the non-adrenalectomized group, even though the survival time of the adrenalectomized group was longer. Once again, the adrenalectomized group treated with adrenal cortical extract fell into an intermediate position. The difference between the adrenalectomized and control animals was significant ($p<0.01$); those between the group given adrenal cortical extract and the other two were not ($p>0.10$).

When the rate of urea synthesis in the nephrectomized rats was compared with that of the nephrectomized-adrenalectomized rats, as shown in Fig. 1, no difference was found initially, but as the experiment progressed increasing and significant differences were found. There was a significant rise in the rate of urea formation in animals with intact adrenals, but the apparent fall in the adrenalectomized animals' rate was not significant.

Discussion. By depleting rats of potassium, it has been possible to maintain life long enough to study their rate of urea formation after adrenalectomy and nephrectomy. Two methods of potassium depletion are available; the use of the low-potassium diet, and the administration of 2 mg of desoxycorticosterone acetate per day for periods of 3 weeks.[1] We have elected to use the special diet because it is more convenient and less expensive than is desoxycorticosterone. It is probable that the high mortality noted in the group of

rats on the chow diet was a result of an inadequate preoperative preparation with desoxycorticosterone, since at this dose level the period of preparation was too short to effect *potassium depletion.*

The 3-day preparatory period we have used probably does not achieve a maximal protective effect, since Durlacher and Darrow's rats[1] prepared with a similar diet for 21 days survived an average of 112 hours as compared to 77 hours for nephrectomized rats with intact adrenals in our series.

Selye and Nielsen[3] have reported increased survival time in rats pretreated with 10 mg of desoxycorticosterone acetate for only 3 days before nephrectomy. In their series, however, the increased survival time may have been due to the functional adrenalectomy produced by such large doses of desoxycorticosterone. The rate of urea formation of the pretreated animals was lower than that of the controls, suggesting a general depression *of adrenal cortical function in this experiment.*

It is of interest that, after potassium depletion, adrenalectomized rats survive nephrectomy for longer periods than do rats with intact adrenals. This is the only instance of which we are aware in which the adrenalectomized animal is more resistant to a stress, *i.e.* nephrectomy and fasting, than is the animal with intact adrenals. It should be emphasized, however, that the adrenalectomized potassium-depleted rat *is not* more resistant to uremia, since the blood urea nitrogen level at the time of death was lower

3 Selye, H., and Nielsen, K., PROC. SOC. EXP. BIOL. AND MED., 1941, 46, 541.
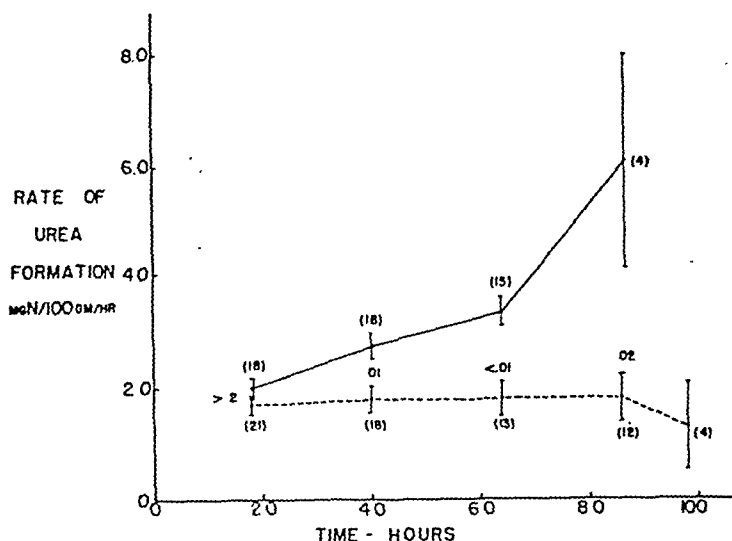
Fig. 1

The rate of urea nitrogen formation per 100 g body weight per hour after nephrectomy in the control (solid line) and adrenalectomized (dashed line) rats maintained on a low potassium diet for 3 days before nephrectomy. The numbers in parentheses refer to the number of observations at each point. The vertical bars represent the standard error of the mean of each point and the numbers between the curves the p values at each point.

in the adrenalectomized than in the control rats. The administration of adrenal cortical extract shortened the survival time of adrenalectomized rats. These effects can all be explained on the basis of the depression of protein turnover, and consequently, of urea formation observed after adrenalectomy. In the control group, the rising rate of urea production was probably due to the usual protein catabolic response to stress which occurs in the presence of the adrenal cortex. Harrison and Long[1] found that the rate of nonprotein nitrogen excretion increased after the second day of fasting in normal rats. The increasing difference in the rate of urea production between the adrenalectomized-nephrectomized and normal nephrectomized rats in our experiment is of the same degree of magnitude as found by Harrison and Long in their studies on nitrogen excretion. Thus

starvation itself may be a sufficient explanation for the rising rate of urea production. The metabolic adjustments of uremia may play little or no part in accelerating urea formation in the nephrectomized rat with intact adrenals.

Summary. Adrenalectomized-nephrectomized rats fed a chow diet before operation have a high mortality rate. If the animals are prepared preoperatively for 3 days on a low-potassium diet, the adrenalectomized animals survive longer than rats with intact adrenals. The rate of production of urea is normal immediately after adrenalectomy, and fails to accelerate in the manner characteristic of rats which intact adrenals subjected to starvation and uremia. The blood urea nitrogen level at death is lower in adrenalectomized than in control animals. Administration of adrenal cortical extract to adrenalectomized-nephrectomized rats tends to restore the survival time and terminal blood urea nitrogen level to control values.

[1] Harrison, H. C., and Long, C. N. H., *Endocrinology*, 1940, **26**, 971.

15999

Attempts to Produce Acute Cardiac Failure by Posterior Pituitary Extracts.

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The counterpart of clinical cardiac failure which develops after prolonged left ventricular strain as a result of working against an elevated arterial pressure has probably not been produced acutely in animal experiments. Until this is accomplished the fundamental cardiodynamic mechanisms involved will not have been fully elucidated. It remains problematical, for example, whether the typical *congestive heart failure which often develops* clinically can occur as a direct consequence of prolonged strain of the left ventricle and reduction in its blood supply, or whether it is due to secondary causes. In the search for a method for producing hypertensive cardiac failure rather acutely, the use of posterior pituitary principles suggested itself. Extracts of the posterior pituitary gland containing chiefly or solely the pressor principles, when injected into anesthetized animals, cause an elevation of arterial pressure by peripheral constriction, constriction of coronary vessels of an intense degree, and probably as a result of such action depression of the myocardium. Pulsus alternans which becomes significant in view of the cardiac slowing induced has also been reported. According to Starling's conception, myocardial failure means dynamically that the ventricles require a greater diastolic distention and higher initial tension in order to expel the same stroke volumes. Indeed, it is by virtue of such depressive action on the ventricles that caution has been urged in the clinical use of posterior pituitary preparations.

Cardiac Effects from Continued or Repeated Administration of Posterior Pituitary Principles. Fourteen dogs weighing from 10 to 18.5 kilos were anesthetized with morphine sulfate and sodium barbital. Aortic pressure

pulses were recorded by a calibrated Gregg optical manometer of adequate sensitivity and frequency. As a first approach, central venous pressures were measured by inserting a glass cannula into the superior vena cava via an external jugular vein and connecting it with the saline manometer, the pressure readings being made, after flushing, during expiration. In some experiments peripheral venous pressures were recorded as well. It was realized that this is a crude procedure and quite inadequate for dynamic studies, but it was sufficient to determine whether a pronounced rise of venous pressure such as is evident during clinical types of decompensation occurred. A number of different pituitary preparations were used and we were not able to detect any special difference between them.* In different experiments pituitary extracts were injected in two different ways, namely, by administration of fractional doses of 2 to 5 P.U. with an occasional administration of a larger dose and by slow continuous intravenous injection of a dilute solution over a period of hours. In the latter a solution containing 1 pressor unit per 10 cc was injected into a femoral vein by use of a Mariotte burette.

Results. Trials with different preparations revealed that a prolonged and marked hypertension cannot be realized from the use of pituitary extracts alone. This was due in a certain measure to the development of an apparent refractoriness to repeated injections of the extracts. Elevation of pressure to high levels was also prevented by the cardiac slowing which supervened and the myocardial depression which accompanied this slowing. However, at the moderate elevations of pres-

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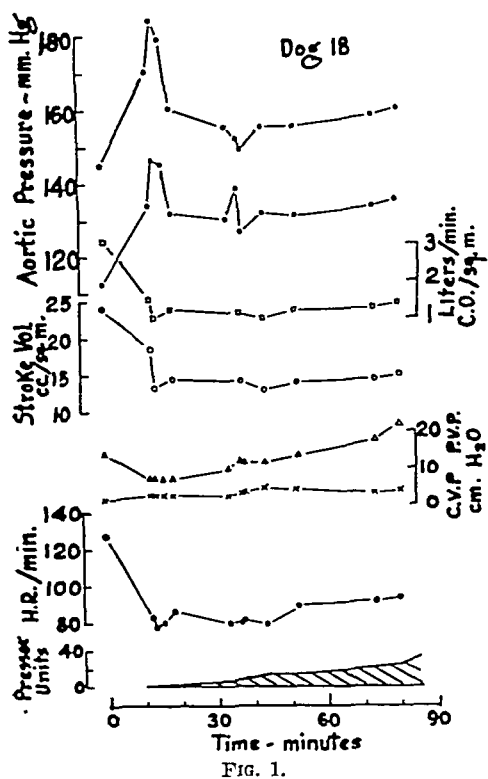
FIG. 1.

sures no consistent elevation of superior vena cava pressure was found to occur which in any way could be interpreted as congestive heart failure according to clinical standards.

Fig. 1 illustrates the main effects of a continuous accelerating infusion.[‡] After a preliminary large rise of systolic and diastolic pressures to a maximum of 187/146 mm Hg (2 upper plots) these pressures decrease to somewhat lower ranges (average 155/132 mm Hg) owing partly to the marked retardation of the heart from 128 to about 80 beats per minute. Estimation of cardiac output by analysis of aortic pressure pulses after the method of Hamilton and Remington[2] indicates that the high arterial pressures are maintained despite the large reduction in stroke volume and cardiac output. Since the stroke volume is decreased despite the marked cardiac slowing it is apparent that the myocardium of

the left ventricle is definitely depressed. This accords with observations previously reported by Wiggers[3] with regard to similar action on the right ventricle. Despite these changes, central venous pressure was not increased materially, although peripheral venous pressures measured by a catheter in the iliac veins rose moderately.

The nature of the myocardial depression induced by continuous action of posterior pituitary principles is revealed by a study of the aortic pressure pulses. These underwent changes in contour which were fairly constant and are exemplified by 8 segments in Fig. 2. Segment 1 is a normal control. Segment 2 shows an initial pressor effect, and segment 3 a subsequent depressor effect following an injection of 2 units of pitressin. Segment 4 represents the recovery but with the slowing maintained after this injection. Obviously, the effects of a single dose of posterior pituitary principles on rate persist longer than those causing depression of the myocardium. It is observed in segments 2 and 3 that regardless of whether the diastolic pressure rises or falls the pulse pressure is materially decreased. The isometric contraction phase indicated by the interval between the small preliminary vibration and the rise of the pressure pulse is definitely prolonged. Some recovery is noted in segment 4. The curve rises much more gradually to a summit in segments 2 and 3, indicating that the velocity of systolic discharge is reduced and its volume decreased. The magnitude of the change in stroke volume estimated according to the method of Hamilton and Remington is indicated directly on the record (S.V.). It will be noted that although the systolic discharge or stroke volume is restored in segment 4 the minute volume remains decidedly reduced owing to the continued reduction in heart rate (HR). Segments 5 to 8 indicate reactions during a later phase of the experiment after administration of pitressin had been discontinued for some time. Segment 5 shows some characteristics of pituitary action after another injection made after the curves of segment 4

‡ Indicated by cross-hatched lower graph.

2 Hamilton, W. F., and Remington, J., Am. J. Physiol., 1947, 148, 14.

3 Wiggers, C. J., Am. J. Physiol., 1914, 33, 382.
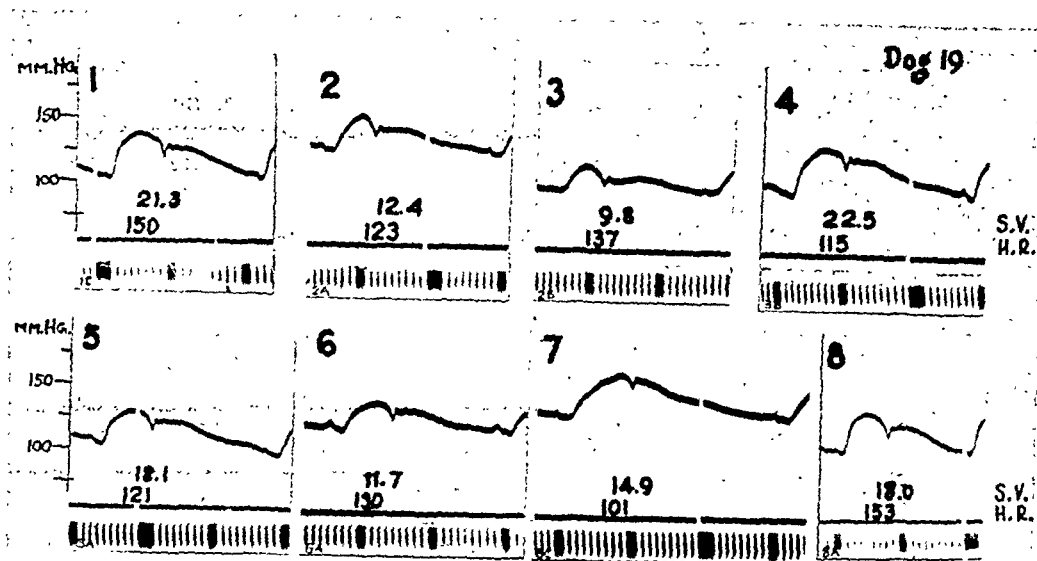
FIG. 2.

had been recorded. Segments 6 and 7 indicate additional and progressive effects of a continuous infusion. It will be noted that the stroke volumes and velocity of ejection by the left ventricle reduce significantly and that in segment 7 the stroke volume was diminished, although the pulse pressure was increased. Segment 8 shows considerable recovery, 52 minutes after the infusion was stopped.

Effects of Pituitary Extracts Plus Additional Hypertension. Since a sufficiently intense hypertension could not be produced by use of pituitary preparations alone, the technic was modified in two ways in an effort to make the left ventricle work against higher arterial pressure levels. In 4 dogs the carotid sinuses were excised and the vagi nerves cut. Thereupon, pitressin or pituitary extracts were again administered. Blood pressure was generally elevated at the start in such animals, although it tended to decline before pituitary extracts were administered. Progressive administration of pitressin either by continuous injection or given in stepwise doses failed to cause a pronounced elevation of arterial pressure required except very large doses which often proved treacherous. The observation was frequently made that after such de-

afferentation the heart rate, instead of decreasing, generally accelerated to an extreme degree. The mechanism remains enigmatic. The conclusion was reached that this also was not always a successful method for reproducing hypertensive cardiac failure acutely. *A second modification consisted in opening the chest, maintaining a mild artificial respiration, and compressing the aorta to various degrees.* This maneuver combined with injection of posterior pituitary extracts enabled us to produce higher systemic pressures although it involved certain complications as well. For example, too great compression of the aorta led to circulatory changes in the abdomen which tended to stagnate blood in the abdominal viscera. For this reason, such compression could not be maintained for too long a time. The typical responses following 3 periods of continued injection of pituitary extracts over a period of 3 hours are indicated in Fig. 3. Arterial pressures were maintained at rather high diastolic levels throughout, the heart rate became progressively slower, but central venous pressures remained remarkably constant. However, this may perhaps be accounted for in part by the reduction in venous return during aortic compression. Right ventricular failure in the clinical sense was
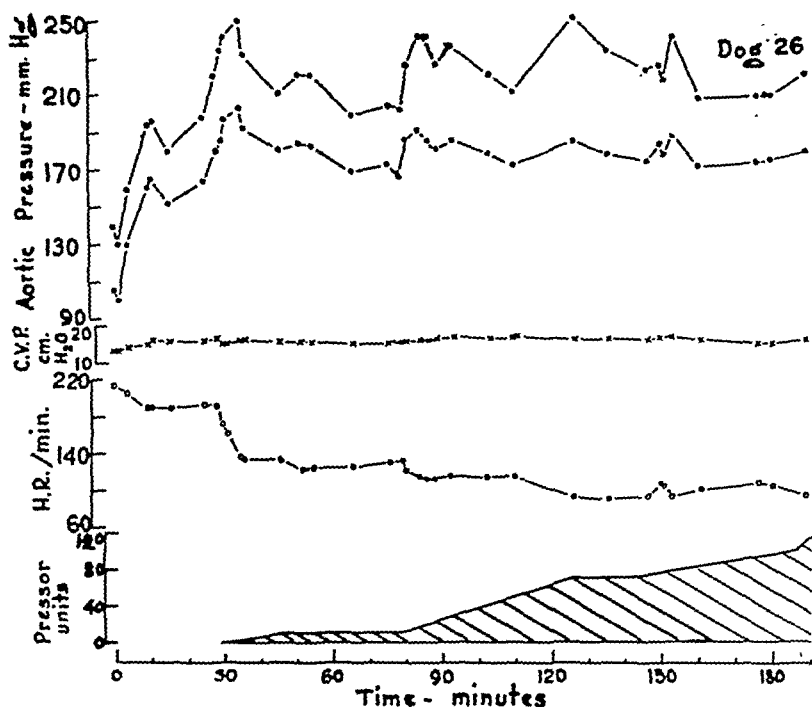
FIG. 3.

certainly not manifested in these experiments.

It was apparent after many trials that the congestive heart failure does not eventuate in dogs when a depressed left ventricle is required to work for 3 to 4 hours against high arterial resistance at the same time that the myocardium of the right heart is depressed and the coronary vessels are constricted. To assure ourselves that posterior pituitary preparations injected into a femoral vein is in sufficient concentration in the blood stream to reduce coronary blood flow while pressures are elevated, experiments were carried out in which flow in the left ramus descendens was measured either by the Opdyke flowmeter[4] or by a bubble flow meter. These experiments showed that significant reduction of blood flow occurred.

The conclusion was reached that if left ventricular strain accompanied by drastic reduction of coronary flow leads directly to cardiac decompensation the time factor must play an important role. Incidentally, our

observations give some reassurance that while posterior pituitary preparations do induce coronary constriction and myocardial depression neither of these actions is apt to cause serious consequences when such preparations are administered in therapeutic doses to patients with normal coronary circulation and cardiac action. Careful continuous administration of 185 units during a period of 3 hours did not lead to permanent damage (Fig. 3).

Summary. An attempt was made to reduplicate clinical conditions in which a depressed left ventricle with a reduced coronary flow is required to work against high arterial pressures (a) by repeated or continuous infusions of posterior pituitary extracts, and (b) by creating additional arterial resistance through cutting of afferent moderator nerves or mechanical compression of the aorta. In all of these ways it proved impossible over periods of 3 to 4 hours to induce a state comparable to clinical congestive heart failure. If such failure is secondary to left ventricular strain plus reduction of coronary flow and

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[4] Opdyke, D. F., and Foreman, *Am. J. Physiol.,* 1947, 148, 726.

myocardial depression, the time element must play a dominant role.

Additional information regarding the nature of left ventricular depression induced by large doses of posterior pituitary extracts was obtained by an analysis of the aortic pressure pulses. Experimental evidence indicated that serious cardiac consequences are not apt to result from use of posterior pituitary preparations administered to patients with normal hearts in therapeutic doses.

16000 P

Effect of Estrogens on Early Development of Frog Embryos.[*]

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Boell[1] showed that cytochrome oxidase activity undergoes changes during the development of *Amblystoma punctatum* embryos, and McShan and Meyer[2] reported that certain estrogens inhibit the succinoxidase system in mammalian tissues. This inhibition by the estrogens was shown to be mediated through the cytochrome oxidase of the system. In the light of these results and the fact that cytochrome oxidase plays an important role in respiration, it became of interest to determine whether exposure of fertilized frog eggs to natural and synthetic estrogens would affect their normal development. Eggs of *Rana pipiens* and *Rana clamitans* were treated with diethylstilbestrol and with estrone in varying concentrations and for varying periods of time. The results of these preliminary experiments will constitute the data presented in this paper.

Frogs were procured from a dealer and stored in the laboratory at 4°C until used. Ovulation was produced and the eggs fertilized according to the standard technique of Rugh.[3]

A 10^{-3} M stock solution of diethylstilbestrol was prepared as reported by McShan and Meyer, and a solution of estrone of the same strength was made by dissolving 2.7 mg of estrone in 0.3 cc of 2 N NaOH plus 0.2 cc of 95% alcohol and diluting to a volume of 10 cc. The required strength of the estrogens was obtained by dilution with 20% Holtfreter's solution.[4] Proper control solutions of NaOH and alcohol were used.

The eggs were treated 1 hour after fertilization (after rotation and before the first cleavage) by dividing them into groups of 30 and placing them in 50 cc of the test solution. After exposure the eggs were rinsed with distilled water, transferred to Holtfreter's solution and maintained at 18°C for 5 days. Stages of development attained by normal and abnormal embryos were designated according to Shumway.[5] Abnormalities were recorded by number from Rugh's series of photographs.[3]

In the first series of experiments eggs of *Rana pipiens* and *Rana clamitans* were exposed to diethylstilbestrol in concentrations of 27, 13.5 and 6.8γ per cc for periods increasing by half hours from ½ hour to 4 hours. Control groups of eggs were placed in 6/100,000, 6/200,000 and 6/400,000 M NaOH and in 20% Holtfreter's solution. In

1 Boell, Edgar J., *J. Exp. Zool.*, 1945, **100**, 331.

2 McShan, W. H., and Meyer, Roland K., *Arch. Biochem.*, 1946, **9**, 165.

3 Rugh, Roberts, *Experimental Embryology*, 1941, N. Y. Univ. Bookstore, 18 Washington Place, New York.

4 Holtfreter, J., *Arch. f. Ent. Mech.*, 1931, **124**, 404.

5 Shumway, Waldo, *Anat. Rec.*, 1940, **78**, 139.

TABLE I.

Effect of Diethylstilbestrol and Estrone on the Development of Eggs of *Rana pipiens*.

Treatment* γ/cc	No. eggs	% abnormal	% survival
D 27.0	154	95.5	12.3
E 27.0	146	100.0	7.5
D 13.5	152	54.6	71.1
E 13.5	152	99.3	6.6
D 6.8	152	34.9	97.4
E 6.8	150	100.0	0
D 2.7	61	4.9	100.0
E 2.7	117	100.0	0.9
E 1.35	123	90.2	26.0
E 0.68	121	26.4	90.1

* Eggs were exposed to the solutions for a period of 2½ hrs beginning 1 hr after fertilization. Development was observed for 5 days.
D = diethylstilbestrol. E = estrone.

R. pipiens, with only one hour's exposure, 27γ of diethylstilbestrol per cc produced approximately 100% abnormal embryos and high mortality; 4 hours with 6.8γ per cc produced less than 40% abnormality and low mortality. 13.5γ per cc for 2½ hours, however, gave approximately 55% abnormality and 30% mortality. Eggs of *R. clamitans* showed a similar variation in response according to concentration and period of exposure but were more sensitive. 1 to 12% of control embryos were abnormal.

On the basis of these results the 2½ hour period of exposure was used for a comparison of the effects of estrone and diethylstilbestrol on eggs of *R. pipiens*. Concentrations ranging from 27 to 0.68γ per cc together with appropriate control solutions were employed. The data in Table I show that the effects obtained with estrone were much greater than those produced by diethylstilbestrol, and that when the ratio of abnormal embryos was about 50% or less the percentage survival was high. Abnormalities in the controls ranged from 1.3 to 10%. The stronger concentrations of both diethylstilbestrol and estrone produced a large number of abnormal embryos which did not develop beyond the blastula stage. Severe exogastrulation and various degrees of spinal bifida were the most common abnormalities observed.

Since it has been shown that estrogens inhibit cytochrome oxidase *in vitro.* further work is being done to determine whether this enzyme system can be inhibited in frog embryos by estrogens and thereby offer a possible explanation for the effects reported herein.

Summary. Abnormalities were produced in the developing eggs of *Rana pipiens* and *Rana clamitans* by exposure to diethylstilbestrol and to estrone in varying concentrations and for varying lengths of time. Higher concentrations of both substances produced *more* abnormalities, and estrone was found to be more effective than diethylstilbestrol.

16001 P

A Simple Method of Producing Control Guinea Pig Immune Sera for Use with Complement Fixing Antigens for the Arthropod-Borne Virus Encephalitides.*

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It was noted in this laboratory that the sera of guinea pigs, following the mild infection resulting from an intracerebral inoculation of St. Louis virus,[1] had good neutral-

izing titers. Later, when complement fixation with this virus and the Japanese B virus was undertaken, it was noted that sera of guinea pigs recovering from infection with either virus, had moderate complement fixing antibody titers. Howitt[2] and later Brown[3] prepared such sera for equine viruses. However, most workers, following the recommendation of Casals and Palacios[4] and of Casals,[5] used hyperimmune mouse sera. Havens et al.[6] used hyperimmune hamster sera. Mice and hamsters have the unique advantage of being susceptible to fatal infections with all members of this group of viruses. Because of this, they can be given a long series of inoculations of any quantity of homologous brain-tissue virus. Disadvantages are that several months of immunization are required to obtain suitable titers; each animal when bled yields a very small quantity of serum, and because of anticomplementary or a non-specific type of reaction inherent in sera from these species, the sera require inactivation at temperatures of 60°C (mice) to 65°C (hamsters). Guinea pigs are susceptible to fatal infection only to the equine viruses. Using guinea pigs in long immunological procedures with mouse brain (other viruses) could be expected to result in sera reacting to all mouse brain antigens. Therefore, even though guinea pig sera require inactivation at only 56°C, they have been avoided in the past.

The following simple technic, however, produces specific sera of high titer in a very short period of time, with minimal work and with a relatively large yield per animal.

Western and Eastern Equine Viruses. 0.5 ml of a suspension of infected guinea pig brain of such dilution (10^{-2} to 10^{-4}) as will produce a paralytic, but non-fatal infection

is given subcutaneously to each of a group of guinea pigs. Ten days later 0.5 ml of a 10^{-2} dilution is given intraperitoneally and after 7 to 10 days, 0.15 ml of a 10^{-1} dilution is given intracerebrally. After 2 weeks the animals are bled to death. The serum is pooled and frozen in CO_2 ice in a large number of small ampoules, or lyophilized in small tubes. The titer of this serum will fall quite rapidly when stored as a liquid at 5°C.

St. Louis, Japanese B, West Nile, Russian Spring-summer and Hammon-Reeves California virus. After a few serial passages in hamsters, ampoules of hamster brain suspension are frozen and stored in CO_2 ice. From the stock source guinea pigs are inoculated. Two intracerebral inoculations are given, 0.15 ml each, of 10% brain suspension at an interval of 10 days. Fever occurs in the guinea pig after the first injection, but rarely are other signs of infection observed. The animals are bled to death 10 or 15 days after the second injection, and the sera preserved as in the case of the equine viruses.

Characteristics of sera. These sera have been tested extensively over a period of several years with many types of brain antigen. A detailed report of the results is being published elsewhere. In no instance has a serum from any of these animals reacted with normal mouse-brain antigen, either before or after these immunization procedures. Japanese B and St. Louis sera usually have titers of 1:256, occasionally 1:512; Eastern equine sera are generally in the range of 1:128 to 1:256, while serum titers to the Western equine and California viruses are of the lowest order, usually 1:64 to 1:128. All are in a range where they can be used in relatively high dilutions as positive controls, or for the standardization of antigens.

When used with mouse-brain antigens made by the Casals technic or by our modification of that of Havens et al. (centrifuged at 16,000 r.p.m.), some minimal overlapping occurs between the Western and Eastern equine sera as reported for monkey and hamster sera by Havens et al., and between the members of the St. Louis-West Nile-Japanese B complex. A slight but less pronounced over-

1 Hammon, W. McD., unpublished data.

2 Howitt, B. F., Proc. Soc. Exp. Biol. and Med., 1937, 35, 526.

3 Brown, C. G., Proc. Soc. Exp. Biol. and Med., 1944, 56, 91.

4 Casals, J., and Palacios, R., J. Exp. Med., 1941, 74, 409.

5 Casals, J., J. Bact., 1945, 50, 1.

6 Havens, W. P., Jr., Watson, D. W., Green, R. H., Lavin, G. I., and Smadel, J. E., J. Exp. Med., 1943, 77, 139.

lapping occurs with sera of the latter group to the California virus antigen, but not in the reverse direction. With DeBoer and Cox[7] benzene extracted antigens, and our recent modification of their method,[8] much less overlapping occurs and when our antigen is employed in its optimal dilution (the highest antigen dilution which gives the highest serum titer in a combined antigen-serum titration),

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[7] DeBoer, C. J., and Cox, H. E., *J. Immunol.*, 1947, 55, 193.

[8] Espana, C., and Hammon, W. McD., PROC. SOC. EXP. BIOL. AND MED., 1947, 66, 101.

there is absolutely no overlapping detectable between any heterologous serum and antigen in original serum dilutions as low as 1:4.

Summary. Herein is described a simple, economical and quick method for preparing highly specific, high titer guinea pig immune sera for use as positive controls, and for standardizing antigens in the complement fixation test. This method has been used for producing sera for Western and Eastern equine, St. Louis, Japanese B, West Nile, Russian Spring-summer and the Hammon-Reeves California virus.

16002

A Suitable Current Stabilizer for the Tiselius Apparatus.*

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It has become evident that present methods of current control for the electrophoretic apparatus of Tiselius can be improved. It is common practice to use a voltage-stabilized power supply to furnish the current. While this power supply gives a constant voltage across the whole cell, it does not furnish a constant current because of fluctuations of resistance within the cell. Most of these fluctuations probably arise within the electrode vessels. Inasmuch as the resistance of the central part of the cell is not a constant fraction of the total resistance of the cell, the voltage drop across this part and the field strength within it do not remain constant. Because of this it is customary[1] to measure the current and to calculate the field strength by means of the equation: $X = I/qk_s$.

X = field strength in volts per cm.

I = current in amperes.

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[1] Abramson, H. A., Moyer, L. S., and Gorin, M. H., *Electrophoresis of Proteins*, p. 61, Reinhold Publishing Co., New York, 1942.

q = cross-sectional area of the central part of the cell.

k_s = specific conductance of sample in reciprocal ohms.

In order to apply this equation to apparatus employing a voltage-stabilized power supply it is necessary for the operator to make frequent observations of the current and to compensate for fluctuations by altering the applied voltage. This frequent adjustment is tedious as well as time-consuming.

It is preferable that current-stabilization rather than voltage-stabilization be used. The simple circuit which has been found suitable for the Tiselius apparatus is herein given. It is based on the principle that the plate current of a pentode vacuum tube remains constant despite changes in plate voltage. Further stabilization is obtained by the degenerative effect of the resistance in the cathode circuit.

In operation the switch S_2 is connected to the dummy load R_3 and then the switch S_1 is closed. This dummy load serves two purposes: first, the tubes are brought to normal operating temperatures in order to avoid subsequent fluctuations due to thermal changes; and secondly, the dummy load has approxi-

izing titers. Later, when complement fixation with this virus and the Japanese B virus was undertaken, it was noted that sera of guinea pigs recovering from infection with either virus, had moderate complement fixing antibody titers. Howitt[2] and later Brown[3] prepared such sera for equine viruses. However, most workers, following the recommendation of Casals and Palacios[4] and of Casals,[5] used hyperimmune mouse sera. Havens et al.[6] used hyperimmune hamster sera. Mice and hamsters have the unique advantage of being susceptible to fatal infections with all members of this group of viruses. Because of this, they can be given a long series of inoculations of any quantity of homologous brain-tissue virus. Disadvantages are that several months of immunization are required to obtain suitable titers; each animal when bled yields a very small quantity of serum, and because of anticomplementary or a non-specific type of reaction inherent in sera from these species, the sera require inactivation at temperatures of 60°C (mice) to 65°C (hamsters). Guinea pigs are susceptible to fatal infection only to the equine viruses. Using guinea pigs in long immunological procedures with mouse brain (other viruses) could be expected to result in sera reacting to all mouse brain antigens. Therefore, even though guinea pig sera require inactivation at only 56°C, they have been avoided in the past.

The following simple technic, however, produces specific sera of high titer in a very short period of time, with minimal work and with a relatively large yield per animal.

Western and Eastern Equine Viruses. 0.5 ml of a suspension of infected guinea pig brain of such dilution (10^{-2} to 10^{-4}) as will produce a paralytic, but non-fatal infection

is given subcutaneously to each of a group of guinea pigs. Ten days later 0.5 ml of a 10^{-2} dilution is given intraperitoneally and after 7 to 10 days, 0.15 ml of a 10^{-1} dilution is given intracerebrally. After 2 weeks the animals are bled to death. The serum is pooled and frozen in CO_2 ice in a large number of small ampoules, or lyophilized in small tubes. The titer of this serum will fall quite rapidly when stored as a liquid at 5°C.

St. Louis, Japanese B, West Nile, Russian Spring-summer and Hammon-Reeves California virus. After a few serial passages in hamsters, ampoules of hamster brain suspension are frozen and stored in CO_2 ice. From the stock source guinea pigs are inoculated. Two intracerebral inoculations are given, 0.15 ml each, of 10% brain suspension at an interval of 10 days. Fever occurs in the guinea pig after the first injection, but rarely are other signs of infection observed. The animals are bled to death 10 or 15 days after the second injection, and the sera preserved as in the case of the equine viruses.

Characteristics of sera. These sera have been tested extensively over a period of several years with many types of brain antigen. A detailed report of the results is being published elsewhere. In no instance has a serum from any of these animals reacted with normal mouse-brain antigen, either before or after these immunization procedures. Japanese B and St. Louis sera usually have titers of 1:256, occasionally 1:512; Eastern equine sera are generally in the range of 1:128 to 1:256, while serum titers to the Western equine and California viruses are of the lowest order, usually 1:64 to 1:128. All are in a range where they can be used in relatively high dilutions as positive controls, or for the standardization of antigens.

When used with mouse-brain antigens made by the Casals technic or by our modification of that of Havens et al. (centrifuged at 16,000 r.p.m.), some minimal overlapping occurs between the Western and Eastern equine sera as reported for monkey and hamster sera by Havens et al., and between the members of the St. Louis-West Nile-Japanese B complex. A slight but less pronounced over-

1 Hammon, W. McD., unpublished data.

2 Howitt, B. F., PROC. SOC. EXP. BIOL. AND MED., 1937, 35, 526.

3 Brown, C. G., PROC. SOC. EXP. BIOL. AND MED., 1944, 56, 91.

4 Casals, J., and Palacios, R., J. Exp. Med., 1941, 74, 409.

5 Casals, J., J. Bact., 1945, 50, 1.

6 Havens, W. P., Jr., Watson, D. W., Green, R. H., Lavin, G. I., and Smadel, J. E., J. Exp. Med., 1943, 77, 139.

16003

Clotting Defect in Hemophilia: Deficiency in a Plasma Factor Required for Platelet Utilization.

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Previous studies[1] have shown that in hemophilic blood there is a delayed conversion of prothrombin into thrombin, apparently because liberation of thromboplastin from the formed elements of the blood is slow. Following removal of formed elements by centrifugation, normal plasma showed a delayed prothrombin conversion rate, but the delay was much less than in hemophilic blood. It was postulated that further centrifugation would produce in normal blood a slow clotting time and a delayed prothrombin conversion rate identical with those in hemophilic blood. It was suggested also that normal plasma, rendered spontaneously incoagulable by freeing it of its formed elements, was needed for a crucial test of the clotting defect in hemophilia.

Fuchs[2] reported having obtained spontaneously incoagulable human plasma by high speed centrifugation of carefully collected blood in paraffined glassware. Neither Feissly[3] nor Smith, Warner and Brinkhous[4] were able to confirm this work, despite the fact that equipment of Fuch's design was used. Recently Jaques and coworkers[5] reported the use of non-wettable surfaces treated with silicone to delay the clotting time of normal blood. In the experiments reported below this method has been adapted to obtain from normal individuals plasmas which clotted slowly or not at all in contact with *ordinary* glass. Such plasmas may be termed quasi-

hemophilic since, like hemophilic plasma, they had a delayed prothrombin conversion rate but clotted promptly after addition of thrombin or thromboplastin. They were tested for their corrective effect on the clotting of true hemophilic blood and plasma. A preliminary report of these data was made recently.[6]

The technic used to obtain quasi-hemophilic and hemophilic plasmas was briefly as follows: Syringes, needles and glassware were treated with a methylchlorosilane, General Electric Dri-Film.[5] Blood was drawn from the antecubital vein directly into sodium citrate solution (3.2% $Na_3C_6H_5O_7 \cdot 2H_2O$) in the ratio of 8 ml blood to 1 ml anticoagulant. The blood was centrifuged initially up to 30 minutes in an angle centrifuge at 5700 r.p.m. (about 3300 g). The supernatant plasma then was subjected to a series of recentrifugations of 30-90 minutes each at the same speed. In instances in which the total centrifugation time exceeded 2 hours, the plasma was centrifuged for a period of 10-20 minutes at 14,000 r.p.m. (about 14,000 g) immediately after the initial centrifugation. Platelet-free plasmas were centrifuged for 165-1350 minutes, platelet-rich plasmas for 5 minutes. Collection and centrifugation of the blood were carried out in a constant temperature room ($2°C$).

Clotting time determinations were made after recalcification with $CaCl_2$ (1.2%) at $28°C$ in ordinary glassware. Each clotting tube contained 0.15 ml citrated plasma. Calcium, sufficient to give the shortest clotting time, and saline (0.9% $NaCl$) were added to make a total volume of 0.25 ml. In testing its effect on the clotting of hemophilic samples, the normal plasma was added in the ratio of 1 part to 10 parts hemophilic plasma. The

[1] Brinkhous, K. M., *Am. J. Med. Sci.*, 1939, 198, 509.

[2] Fuchs, H. J., *Arch. exp. Zellforsch.*, 1933, 14, 334.

[3] Feissly, R., *Helv. Med. Acta*, 1940, 7, 583.

[4] Smith, H. P., Warner, E. D., and Brinkhous, K. M., unpublished data.

[5] Jaques, L. B., Fidlar, E., Feldsted, E. T., and Macdonald, A. G., *Canadian Med. Assn. J.*, 1946, 55, 26.

[6] Brinkhous, K. M., *Fed. Proc.*, 1947, 6, 389.

To Electrophoresis Cell

110 V. A.C.
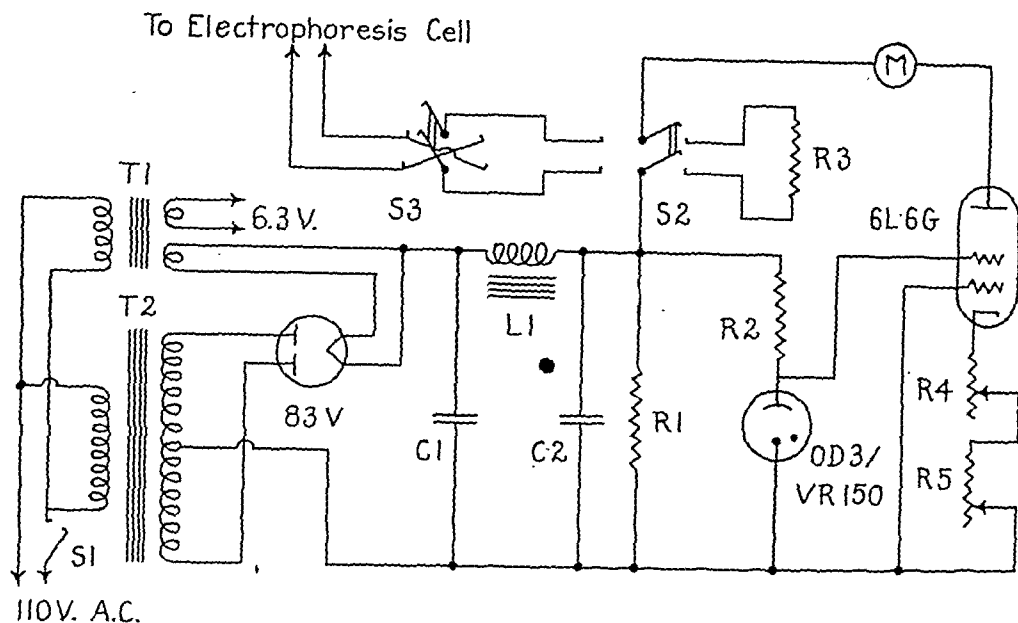
PARTS LIST

T1—Filament transformer with 5 V and 6.3 V windings.
T2—Power transformer—450 V each side of center tap.
L1—Filter choke—10 h.
C1, C2—Filter condensors—8 mfd, 600 V.
R1—50,000 ohms, 10 watts.
R2, R3—20,000 ohms, 50 watts.
R4—Potentiometer rheostat, 2000 ohms, 5 watts.
R5—Potentiometer rheostat, 200 ohms, 5 watts.
S1—S. P. S. T. switch.
S2, S3—D. P. D. T. switches.
M—0-30 milliameter, accurate to 0.5%.

mately the same resistance as the electrophoretic cell and so the 2,000 ohm rheostat R4 may be set so that the current is approximately that required in the cell. Then S2 is changed so that the current will flow through the cell and the final adjustment made with the 50 ohm rheostat R5. S3 is a reversing switch employed for alternation of the polarity of the electrodes on successive runs. The switches and controls are mounted so that the power switch S1 is never closed when the cathode resistance is very small or when the plate circuit of the 6L6 G is open. This avoids damage to the tube.

It is found that the current variations with this source are undetectable on the milliammeter (½% accuracy) used to measure the current. The effect of fluctuations of line voltage and of cell resistance is negligible.

Thus the current through the Tiselius cell is maintained at a constant value. The measurement of current by means of a standard resistance and potentiometer seems unnecessary and unjustified. A precise milliammeter is much more convenient and inexpensive and offers sufficient precision for all electrophoretic studies.

The cost of this circuit, exclusive of the milliammeter, was less than 20 dollars. For size, cost and performance it has been satisfactory for currents of 15-30 ma. It is possible that some of the simple constant-current circuits studied by Hill[3] could easily be adapted for use with electrophoretic cells.

2 Swingle, S. M., Rev. Sci. Inst., 1947, 18, 128.

3 Hill, W. R., Proc. of the I. R. E., November, 1945, p. 785.

TABLE II.
Effect of Addition of Normal Blood or Plasmas on Clotting of Hemophilic Blood or Plasmas.

	Clotting time Recalcified hemophilic blood or plasma, after addition of normal plasma	
Clotting time Recalcified normal plasma (to be added to hemophilic plasma), min.	Whole hemophilic blood, min.	Platelet-free hemophilic plasma, min.
6*	6	6
8	7	18
17	6½	19
31	6	26
40	7¾	30
no clot 1800	6	no clot 1800
	45-120†	no clot 1800†

* Whole blood.
† Controls, showing clotting times of various hemophilic specimens after recalcification, without normal plasma addition.

TABLE III.
Effect of Platelet Suspensions and Normal Plasma* on Clotting of Platelet-free Hemophilic Plasma.*

Supplement	Clotting time Recalcified hemophilic plasma, after addition of platelets or plasma, min.
Normal platelet suspension	32
Hemophilic platelet suspension	28
Normal platelet-poor plasma†	30
Normal plasma + normal platelet susp.	8½
Normal plasma + hemophilic platelet susp.	6

* Plasmas used in this experiment had been centrifuged for 120 minutes.
† Clotting time of recalcified normal plasma was 32 minutes.

platelet-free hemophilic plasma (see Table III). With the platelets alone the clotting time remained prolonged, but in the presence of normal plasma, which by itself did not correct the clotting defect, the platelets reduced the clotting time to the normal range.

Discussion. The data indicate that the clotting of normal plasma depends upon the presence of formed elements. Plasma deprived of formed elements or their disintegration products is stable, even in the presence of a wettable surface and optimal calcium in concentration. The ability to clot is restored by addition of platelets. Unless formed elements are present, normal stable plasma is without effect on the clotting of hemophilic plasma.

These data indicate that platelets and perhaps other formed elements cannot be utilized in the absence of a plasma factor present in normal blood.

A simple hypothesis based on these findings is offered: Normal plasma contains a factor necessary for thromboplastin liberation from the formed elements of the blood, presumably by platelet lysis. This platelet-lysin or thrombycytolysin is deficient in hemophilic plasma. When the lytic factor is supplied to hemophiliacs, as in blood or plasma transfusions, platelets rupture in the normal manner. Sufficient thromboplastin then becomes available, and the block in the clotting mechanism is removed.

Numerous workers, including Feissly,[9] have attempted to test the effect of normal plasma on hemophilic plasma in the absence of platelets. Their results have shown an acceleration of the clotting of hemophilic plasma in the apparent absence of platelets. These discordant results may be due to the presence of free thromboplastin in the normal plasma from breakdown of the formed elements during collection and handling of the plasma. and perhaps also from incomplete removal of formed elements by centrifugation.

In the experiments presented in this paper, the centrifugal forces used were so low that it appears unlikely that any macromolecular substance essential for clotting, such as the thromboplastic protein described by Chargaff

9 Feissly, R., Helv. Med. Acta, 1945, 12, 467.

TABLE I.
Effect of Centrifugation Time on Clotting of Recalcified Normal Blood or Plasma.

Centrifugation time min.	Clotting time min.
0	6½
10	8½
30	10
60	18½
90	20
120	31
165	37
1350	no clot 1800

actual volume added was adjusted to correct for the citrate content. When whole blood was used, the volume was adjusted in addition for cell volume as indicated by the hematocrit.

Platelet suspensions were prepared by differential centrifugation of blood in silicone glassware. The platelet sediment was washed by resuspension in citrate-saline solution (1 part citrate solution, 9 parts saline) and recentrifuged. This was repeated 3 times. Microscopic examination showed the platelets to be well preserved. Platelet suspensions were added to provide 25,000-30,000 platelets per mm^3 in the final clotting mixture.

Most of the data on hemophilic blood was obtained from one patient, although the experiments were repeated on at least one of 3 other hemophilic patients. The normal blood samples were obtained from 7 different individuals.

Quasi-hemophilic plasma from normal blood. Table I shows that centrifugation of normal blood for ½ hour or less produced plasmas with clotting times within the range commonly accepted as normal, but that longer centrifugation produced progressively longer clotting times. Finally, plasma was obtained which did not clot within 30 hours after recalcification.

The prothrombin content[7,8] of several samples of quasi-hemophilic plasma, including those spontaneously incoagulable, was within the normal range of 90% to 110%. The prothrombin conversion rate of these samples, determined as previously described,[1] was delayed. The longer the clotting time, the greater was the delay.

Plasmas, similar to those shown in Table I, and whole blood were clotted with the same rapidity by thrombin solutions (Parke Davis "Thrombin Topical") and by thromboplastic extract of beef lung.[8] Tests for antithrombin activity gave normal values. All of the plasmas, when incubated with thrombin solutions, destroyed thrombin in the same amount and at the same rate.

The addition of platelet suspensions to the quasi-hemophilic plasmas reduced the clotting time to 6 to 9 minutes. This is in contrast to the behaviour of platelet-free hemophilic plasma described later (see Table III).

Failure of quasi-hemophilic plasma to correct the clotting defect of platelet-free hemophilic plasma (see Table II). Normal citrated whole blood reduced the clotting times both of whole hemophilic blood and of platelet-free hemophilic plasma to 6 minutes. The effectiveness of the normal plasmas in correcting the clotting defect of platelet-free hemophilic plasma decreased as their clotting times became prolonged. The spontaneously incoagulable normal plasma was entirely without effect. Regardless of the degree of prolongation of their clotting times, however, the normal plasmas retained their corrective action on the clotting of whole hemophilic blood.

Corrective action of normal plasma on the clotting of hemophilic plasma in the presence of platelets. In a series of further tests, quasi-hemophilic plasma was added to platelet-rich hemophilic plasma, and platelet-rich normal plasma was added to platelet-free hemophilic plasma. In the presence of numerous platelets, either normal or hemophilic, the hemophilic clotting time was reduced to the normal range. The results of one test follow. When a sample of platelet-poor normal plasma which had a clotting time of 27 minutes was added to platelet-free hemophilic plasma, the clotting time was 25 minutes; when it was added to platelet-rich hemophilic plasma the clotting time was 7 minutes, and when added to whole hemophilic blood, clotting occurred in 7½ minutes.

Effect of platelet suspensions on clotting of

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7 Warner, E. D., Brinkhous, K. M., and Smith, H. P., *Am. J. Physiol.*, 1936, **114**, 667.

8 Smith, H. P., Warner, E. D., and Brinkhous, K. M., *J. Exp. Med.*, 1937, **66**, 801.

ent at the beginning of the experiment.

Results of a representative experiment are presented in Table I. Concentrations up to 200 units per cc produced little or no inhibition of growth when no further penicillin was added. When penicillin was added at 3 day intervals, inhibition was noted in the tube originally containing 200 units per cc, indicating that penicillin inactivation was relatively slight, and that the final concentration was considerably in excess of 200 units per cc. With concentrations above 600 units per cc there was not only inhibition, but an actual decrease in optical density, indicative of lysis of the organisms.

A secondary outgrowth of the inhibited and partly lysed organisms was observed in the tubes containing 600 and 800 units per cc, when no more penicillin was added, but no secondary outgrowth occurred with a concentration of 1000 units per cc.

Relation of Inoculum Size and Medium to Penicillin Inhibition. Tubes containing 5 cc of liquid Tween-albumin or oleic acid-albumin media with various concentrations of penicillin were inoculated with tenfold dilutions of a 7-day-old culture of H37Rv diluted in 0.5 cc of distilled water. The inoculum for one set of tubes had been grown in Tween-albumin medium (dispersed growth), and for another set in oleic acid-albumin medium (granular growth). To allow for possible penicillin inactivation, 2 sets of tubes were inoculated. To one set, penicillin in the original concentrations was added on 3 occasions at 3-day intervals; to another no further penicillin was added to the amounts present at the beginning of the experiment. The results were recorded after 14 days incubation, complete inhibition of growth being regarded as the endpoint.

As shown in Table II, the inhibitory effect of penicillin was much greater in the Tween-albumin medium than in the oleic acid-albumin medium, and this difference was related to the size of the inoculum. In the oleic acid-albumin medium, growth was inhibited by 1000 units per cc; with 100 units per cc, no

TABLE I.
Bacteriostasis and Lysis of a Large Inoculum of Tubercle Bacilli by Penicillin in Tween-Albumin Medium.

A. Original Amounts of Penicillin Added 4 Times at 3-day Intervals.

Days	1	4	8	13	17
Penicillin concentrations, units per cc					
0	.082*	.156	.252	.366	.41
100	.082	.155	.222	.301	.40
200	.082	.125	.201	.275	.318
400	.082	.082	.061	.061	.061
600	.082	.061	.051	.051	.051
800	.082	.061	.051	.051	.051
1000	.082	.055	.051	.051	.051

B. No Penicillin Added to Initial Concentrations.

Days	1	4	8	13	17
Penicillin concentrations, units per cc					
0	.082*	.156	.252	.376	.43
100	.082	.156	.252	.376	.43
200	.082	.136	.252	.356	.41
400	.082	.102	.214	.346	.41
600	.082	.082	.175	.327	.41
800	.082	.061	.055	.097	.20
1000	.082	.055	.055	.055	.055

* Turbidities (optical densities) were measured with a Coleman spectrophotometer.

inhibition occurred, growth being as good as in the control tubes, regardless of the size of the inoculum. With the Tween-albumin medium, on the other hand, growth in 100 units per cc occurred only with the largest inoculum, and with smaller inocula the amount of penicillin necessary to suppress growth rapidly declined to 1 unit per cc or less.

When the inoculum was grown in the oleic acid-albumin medium, *i. e.*, when it consisted of clumps rather than diffusely growing bacilli, the inhibitory effect of penicillin in the Tween-albumin medium was somewhat less marked. This suggests that the clumps were better able to survive initially in the presence of penicillin. However, the Tween effect was not overcome entirely, possibly because even with clumps as an inoculum, subsequent growth in the Tween-albumin medium was diffuse.

As in the previous experiment, evidence of destruction of penicillin by the tubercle bacil-

and West,[10] could have been removed by the centrifugation.

Summary. 1. By special handling and prolonged centrifugation of normal blood, plasmas with a delayed clotting time can be obtained. If centrifugation is prolonged sufficiently, the plasmas become spontaneously incoagulable.

2. Normal plasmas of this type require the presence of platelets and perhaps other formed elements to correct the delayed clotting of hemophilic plasma.

3. These findings indicate that in hemophilia there is a deficiency in a plasma factor required for platelet utilization. It is suggested that this factor is a *thrombocytolysin.*

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[10] Chargaff, E., and West, R., *J. Biol. Chem.*, 1946, **166**, 189.

<center>16004</center>

Effect of Penicillin on the Tubercle Bacillus *In vitro*.

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Reports concerning the effect of penicillin on the tubercle bacillus are contradictory. The Oxford group originally noted no inhibition of growth in glycerol broth with a penicillin concentration of 40 units per cc.[1] Subsequently, inhibition by as little as 20 units per cc was observed by one investigator,[2] whereas others reported no inhibition with concentrations of 20 to 30 units per cc.[3,4] Actual stimulation of growth by small concentrations of penicillin was described in one instance,[5] while in another, rapid destruction of large amounts of penicillin (800 units per cc) by culture filtrates of *M. tuberculosis* was observed.[6] These contradictory results, and the desirability of using penicillin to suppress the growth of contaminants in cultures for the primary isolation of tubercle bacilli, have made it appropriate to reinvestigate the effect of penicillin on the tubercle bacillus *in vitro*.

Materials. H37Rv, a standard virulent human strain of *M. tuberculosis*, was used throughout the experiments because of its similarity in virulence and response to antimicrobial agents to strains isolated from patients with tuberculosis.[7,8]

The experiments were performed in liquid and on solid media recently developed in this laboratory.[9,10] The penicillin employed was commercial crystalline penicillin G, obtained from various manufacturers.

Experimental. Lytic Action of Penicillin. A large inoculum of tubercle bacilli, 0.25 mg per cc, was added to test tubes 25 by 150 mm each containing 15 cc of Tween-albumin medium and penicillin in concentrations ranging from 100 to 1000 units per cc. The tubes were incubated at 37°C, and optical densities were recorded on the Coleman Spectrophotometer every day or two. Because of the possible destructive action of the tubercle bacillus on penicillin, the original concentrations were added to one set of tubes every 3 days on 4 occasions. To another set, no further penicillin was added to the amounts pres-

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1 Abraham, E. P., *et al.*, *Lancet*, 1941, **2**, 177.

2 Iland, C. N., *J. Path. and Bact.*, 1946, **58**, 495.

3 Smith, M. I., and Emmart, E. W., *Pub. Health Rep.*, 1944, **59**, 417.

4 Friedmann, I., *Tubercle*, 1945, **26**, 75.

5 Ungar, J., and Muggleton, P., *J. Path. and Bact.*, 1946, **58**, 501.

6 Woodruff, H. B., and Foster, J. W., *J. Bact.*, 1945, **49**, 7.

7 Middlebrook, G., and Yegian, D., *Am. Rev. Tuberc.*, 1946, **54**, 553.

8 Feldman, W. H., and Hinshaw, H. C., *Am. Rev. Tuberc.*, 1947, **55**, 428.

9 Dubos, R. J., and Davis, B. D., *J. Exp. Med.*, 1946, **83**, 409.

10 Dubos, R. J., and Middlebrook, G., *Am. Rev. Tuberc.*, in press.

agents against other bacteria by various wetting agents is well known.[13] This increased inhibitory action of penicillin against the tubercle bacillus in the Tween-albumin medium is similar to that recently observed by Fisher with streptomycin.[14]

The relatively slight destruction of penicillin by the human virulent strain H37Rv in the present studies is in contrast to the marked destruction reported by others using nonpathogenic laboratory strains.[2,6] It may be noted that one of these strains, No. 607, which is often used in studies of tubercle bacilli, does not fulfill the cultural and biological requirements of a true tubercle bacillus. It should be stated that since there was relatively little decline in penicillin activity, no attempt was made in the present experiments to determine quantitatively the extent to which the decline was due to inactivation of penicillin by tubercle bacilli, to deterioration in the incubator, or to a secondary outgrowth of resistant variants.

It would appear that the addition of penicillin in concentrations of 50 to 100 units per cc to the liquid or solid oleic acid-albumin medium may prove a valuable adjunct in culturing contaminated materials.

Current methods of treating clinical specimens often do not destroy all of the contaminants, and inhibitory dyes, such as malachite green, which are ordinarily added to the classical egg yolk-potato media, may also inhibit the growth of the tubercle bacillus.[15] It should be realized, of course, that many types of organisms are not inhibited by penicillin, although concentrations as high as 50 to 100 units per cc may be adequate to suppress the growth of many bacteria and fungi not ordinarily considered susceptible.

Summary and Conclusions. Using appropriate experimental conditions, a virulent human strain of *M. tuberculosis* has been found to undergo partial lysis in the presence of high concentrations of penicillin.

Small inocula of tubercle bacilli are highly susceptible to concentrations of penicillin as low as 1 unit per cc in the Tween-albumin medium. In the oleic acid-albumin medium, penicillin in a concentration of 100 units per cc causes no inhibition of growth, even with the smallest inocula.

Preliminary experiments indicate that in both the solid and liquid oleic acid-albumin medium, penicillin in concentrations of 50 to 100 units per cc may prove a valuable adjunct in culturing tubercle bacilli from contaminated materials.

13 McCulloch, E. C., *Disinfection and Sterilization*, 2nd Edition, Lea and Febiger, Phila., 1945.
14 Fisher, M. W., *Am. Rev. Tuberc.*, in press.
15 Corper, H. J., and Cohn, M. L., *Am. Rev. Tuberc.*, 1946, 53, 575.

16005

Some Antibacterial Properties of Mandelamine (Methenamine Mandelate).

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In recent years, the sulfonamide drugs and the antibiotics have dominated the field of chemotherapy. The successful use of these drugs in the treatment of urinary tract infections, however, is dependent upon an unobstructed urine flow plus adequate kidney function. Further, administration of these drugs requires close medical supervision and, finally, invading organisms tend to develop resistance to both classes of drugs. For these reasons, a relatively non-toxic drug which can be administered by mouth over protracted periods of time to ambulant patients would be highly desirable. According to Carroll and Allen[1]

1 Carroll, G., and Allen, H. N., *J. Urology*, 1946, 55, 674.

lus was slight. Except for the first two tubes in the oleic acid-albumin medium, the endpoint was the same whether further penicillin was added or not.

These results were extended to the solid oleic acid-albumin agar medium, on which, even with the smallest inocula (3 or 4 organisms per plate), no inhibition of growth was noted with penicillin in a concentration of 100 units per cc.

In preliminary experiments with contaminated material, quantitative plate counts were made from saline suspensions of the macerated lungs of Swiss mice infected intravenously with H37Rv. The control plates were often so heavily contaminated with other bacteria that the presence or absence of tubercle bacilli could not be determined. Plates containing penicillin in a concentration of 100 units per cc almost invariably showed complete suppression of growth of the contaminants, and in no instance was there evidence of inhibition of growth of the tubercle bacilli.

Comment. The foregoing experiments demonstrate that under appropriate circumstances tubercle bacilli undergo partial lysis in the presence of penicillin. Many other bacteria are lysed by penicillin, some, such as staphylococci, quite rapidly and completely,[11] and others, such as anaerobic streptococci, more slowly and to a lesser degree.[12] Tubercle bacilli appear to fall into the latter category.

The explanation for the much greater inhibitory effect of penicillin in the Tween-albumin than in the oleic acid-albumin medium is not entirely clear, but it may be found in part in the more intimate contact between the antibiotic and the individual organisms in the Tween-albumin medium, in which growth is diffuse, than in the oleic acid-albumin medium, in which growth is granular. In addition, specific alterations in the physico-chemical characteristics of the cell, caused by the presence of the non-ionic, surface active wetting agent "Tween 80," may play a major role in the phenomenon. Enhancement of the activity of antimicrobial

11 Kirby, W. M. M., *J. Clin. Invest.*, 1945, **24**, 165.

12 Todd, E. W., *Lancet*, 1945, **2**, 172.

TABLE II.

Inhibitory Effect of Penicillin on Various Inocula of Tubercle Bacilli in Tween-albumin and Oleic Acid-albumin Media.

Penicillin, units per cc	Inoculum grown in Tween-albumin medium																				Inoculum grown in oleic acid medium									
	Penicillin added at 3-day intervals										No additional penicillin added										No additional penicillin added									
	Tween medium					Oleic acid medium					Tween medium					Oleic acid medium					Tween medium					Oleic acid medium				
Inoculum →	*1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
1000	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	+	+	o	o	o	o	o	o	o	o	+	+	+	+	+
100	+	o	o	o	o	+	+	+	+	+	+	o	o	o	o	+	+	+	+	+	+	+	o	o	o	+	+	+	+	+
20	+	+	o	o	o	+	+	+	+	+	+	+	o	o	o	+	+	+	+	+	+	+	+	o	o	+	+	+	+	+
5	+	+	o	o	o	+	+	+	+	+	+	+	o	o	o	+	+	+	+	+	+	+	+	+	o	+	+	+	+	+
1	+	+	+	o	o	+	+	+	+	+	+	+	+	o	o	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

* 1—2.5-2 mg dry wt. 2—2.5-3 mg dry wt. 3—2.5-1 mg dry wt. 4—2.5-5 mg dry wt. 5—2.5-6 mg dry wt.

TABLE II.

Recently Isolated Strains in Normal Sterile Urine Buffered at pH 5.5 and 6.5. Minimal bacteriostatic and bactericidal concentrations in mg %. Each figure is the average of 5 different series of tests.

pH	Mandelamine 5.5 static	5.5 cidal	6.5 static	6.5 cidal	Streptomycin 5.5 static	5.5 cidal	6.5 static	6.5 cidal	Sulfathiazole 5.5 static	5.5 cidal	6.5 static	6.5 cidal
E. coli Grogan	25	25	62.5	62.5	10	15	5	10	1.56	5	1.25	20
" " Fernande	50	50	150	150	15	15	5	10	10	20	0.6	0.6
" " Travis	50	50	125	125	20	20	5	5	3	10	1.25	>40
A. aerogenes Harrington	30	30	62.5	62.5	5	5	2.5	2.5	20	20	2.5	40
St. albus No. 6	25	50	75	75	20	20	10	15	.625	2.5	1.25	20
" " Agostini	125	—	75	75	40	—	10	20	5	—	1.25	5
St. hem. No. 9	62.5	—	50	50	20	—	20	20	2.5	—	.6	.6
Ps. pyocyaneous No. 7	125	125	200	200	40	40	20	30	5	40	20	40
Ps. aeruginosa Fox	62.5	62.5	200	200	20	20	5	10	2.5	10	2.5	20

TABLE III.

Induced Resistance to Sulfathiazole, Streptomycin, and Mandelamine. Minimal bacteriostatic concentrations of mandelamine before and after development of resistance to sulfathiazole and to streptomycin. Concentrations given in mg %. Each figure is the average of 2 different series of tests.

Organism	Sulfathiazole E. coli G	Aero aero H	S. aur. 209	Streptomycin E. coli 64	Aero aero 129	S. aur. SM	Mandelamine E. coli G	Aero aero H	S. aur. 209	E. coli 64	Aero aero 129	S. aur. SM
Original min. bacteriostatic conc.	20 (ca. 75 mg %)	20	20	50	50	15						
Final min. bacteriostatic conc.	>Sat.	>Sat.	>Sat.	1500	1200	1500						
Increase in resistance	>4X	>4X	>4X	30X	24X	100X						
Number of transfers	5	3	3	8	10	9						
Original min. bacteriostatic conc. of mandelamine							25	100	200	200	200	20
Min. bacteriostatic conc. of mandelamine after induction of resistance							75	300	500	150	75	37.5

TABLE 1.

Stock Laboratory Strains in Normal Sterile Urine Buffered at pH 5.5. Minimal bacteriostatic and bactericidal concentrations of mandelamine, streptomycin, and sulfathiazole given in mg %. Each figure is the average of 5 different series of tests.

	Mandelamine		Streptomycin		Sulfathiazole	
	Static	Cidal	Static	Cidal	Static	Cidal
E. coli 64	50	50	20	20	5	10
,, ,, 4R5190	50	50	20	20	7.5	20
A. aerogenes 129	25	25	20	20	3	5
K. friedlanderi type AF12	12.5	12.5	5	5	5	10
,, ,, ,, BEGS	25	25	5	5	5	10
St. aureus 209	15	25	10	20	2.5	10
,, ,, SM	20	25	40	40	10	10
Ps. aeruginosa ATCC 9029	40	40	40	40	6.25	20

and Kirwin and Bridges,[2] mandelamine appears to be such a drug. In order to obtain an additional evaluation of the drug, the activities of mandelamine, sulfathiazole and streptomycin against organisms commonly found in urinary tract infections were compared, and the development of resistance by representative organisms to all three drugs was studied. The findings are set forth in the following paragraphs.

Experimental. The medium used throughout this work consisted of normal male urine adjusted to pH 5.5 or 6.5 with phthalate and phosphate buffers in .03 to .06 molar concentrations, respectively. The pH was determined before and after incubation by means of a Coleman model 3 pH electrometer. In the presence of effective bacteriostasis, the pH did not vary by more than 0.5 pH units in any experiment. The inoculum was always 0.1 ml of a 1:5000 dilution of an 18-hr. broth culture. Suitable control tubes of buffered and unbuffered nutrient broth, and of buffered urine were included in each test. If any of the control tubes showed no growth, the whole series was discarded. Eight stock laboratory strains of bacteria commonly found in urinary tract infections were studied at pH 5.5 and 9 strains of organisms recently isolated from urinary tract infections were studied both at pH 5.5 and 6.5. Minimal bacteriostatic concentrations of mandelamine, sulfathiazole and streptomycin were determined by means of a serial

2-fold dilution method. End-points were taken as the lowest drug concentrations at which no growth occurred within 72 hours. Bactericidal activity was determined by subculturing 0.25 ml from each apparently negative tube into 5 ml of nutrient broth followed by examination for visible growth after 72 hours.

Attempts were made to induce resistance in 6 different organisms to streptomycin, sulfathiazole and mandelamine as follows: 0.5 ml of an 18-hour nutrient broth culture was used to inoculate 5 ml of sterile urine, at pH 5.5, in the presence of serial 2-fold dilutions of the individual drugs. After 72 hours incubation, 0.5 ml samples were taken from the tubes containing the highest drug concentrations in which growth had occurred. These were transferred into tubes containing a higher series of drug concentrations, and so on. Finally, when organisms had developed resistance to streptomycin or to sulfathiazole, the antibacterial activity of mandelamine against the resistant organisms was determined.

Results and Discussion. The average minimal bacteriostatic and bactericidal concentrations of sulfathiazole, streptomycin and mandelamine at pH 5.5 against the 8 stock laboratory strains are shown in Table I. The results indicate that minor differences in potency exist but, in general, the activities of all 3 drugs are of the same order of magnitude.

The data obtained at pH 5.5 and 6.5 with the 9 strains recently isolated from urinary tract infections are shown in Table II. The results indicate that streptomycin is somewhat less active at pH 5.5 than at 6.5, the difference being related, presumably, to destruction of

2 Kirwin, T. J., and Bridges, J. P., *Am. J. Surg.*, 1941, **52**, 477.
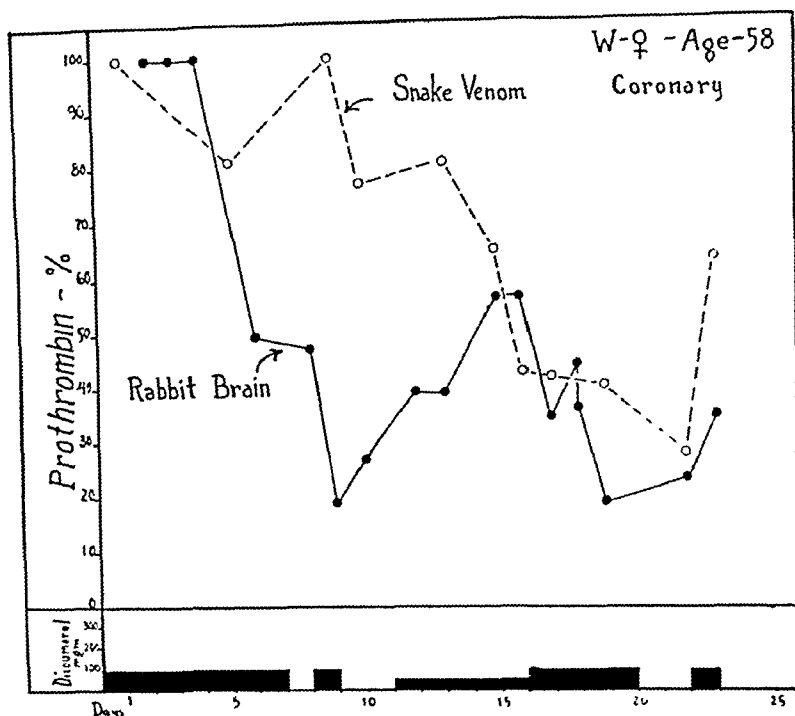
FIG. 1.

Variations in prothrombin during dicoumarol therapy as measured by Quick's method (rabbit brain thromboplastin) and the Russell viper venom modification. Quick's method can be correlated with changes in dicoumarol dosage, the venom method cannot.

of Quick[3] and Page[2] were compared in seven patients receiving dicoumarol therapy.

Striking differences were observed in the quantitative levels of prothrombin as measured by the two methods. Fig. 1 illustrates graphically these variations in a white female who was given dicoumarol orally for coronary occlusion. The therapeutic amounts of dicoumarol, including the initial dosage and changes in the amount of the drug, are closely correlated with the prothrombin levels as determined by the method of Quick, using rabbit brain as the thromboplastin. There is a slow gradual decrease in the prothrombin level as measured by the Russell viper method which cannot be correlated with the Dicoumarol therapy.

Other patients exhibited the same differences. One developed hematuria and it was

observed that the prothrombin was less than 5% of normal by the method of Quick and 61% of normal by the Russell viper venom technique. The results of the two methods were not comparable until 4 days later. In all of the patients observed the prothrombin as determined by the method of Quick decreased to the desired therapeutic levels long before there was a decrease by the snake venom method. The levels of prothrombin as determined by the method of Quick could be correlated with the clinical hemorrhagic state of the patients, whereas the amount of prothrombin as determined by the Russell viper venom method resulted in false safe levels. Frequently the patients received too much dicoumarol because of the results of the venom method.

Conclusions. The quantitative values of prothrombin as determined by the method of Quick using rabbit brain thromboplastin can be correlated with dicoumarol therapy and

[2] Page, R. C., and Russell, H. K., *J. Lab. and Clin. Med.*, 1940, 26, 1366.

[3] Quick, A. J., *J. A. M. A.*, 1938, 110, 1658.

the drug at the lower pH. Sulfathiazole is approximately equally active at both pH levels, but the threat of sulfonamide urolithiasis[3] at the lower level should preclude its use in acidic urine. Mandelamine is more effective at pH 5.5 than at 6.5 but at either pH bactericidal concentrations are low enough to permit ready attainment *in vivo* by appropriate dosing.[1] In connection with this heightened activity at the lower pH values, it is to be noted that mandelamine is an acidifying agent, and Carroll and Allen[1] report, "The urine of all patients (200) in the series but 8 became or remained acid on the therapeutic regimen without other medication or restriction of diet or fluid intake."

As shown in Table III, organisms grew in urine which was saturated, at pH 5.5, with sulfathiazole. The degree of resistance which can be developed is necessarily limited by this restricted solubility of the drug, but within this limitation, a 4-fold increase in resistance occurred after as little as 3 to 5 transfers. Resistance to streptomycin developed more rapidly, an increase of 24- to 100-fold being found after 8 to 10 transfers. In contrast to the foregoing, resistance to mandelamine either did not appear, or if it did, it appeared slowly and only to a limited degree; for ex-

ample, after 9 to 10 transfers there was no change in the susceptibility of 3 organisms, while with the remaining organisms there was only a 3-fold increase in resistance. From the same table, it appears that sulfathiazole-resistant organisms displayed a slightly increased resistance to mandelamine, while the streptomycin-resistant bacteria did not. Further, it appears that mandelamine retained its bactericidal activity against *A. aerogenes H* and *S. aureus* 209, but was only bacteriostatic to the other 4 organisms. These preliminary findings, which suggest that resistance developed against one drug may alter susceptibility to another chemically unrelated drug, are somewhat anomalous, and are being investigated further.

Summary. A comparison of mandelamine, streptomycin and sulfathiazole against 8 stock laboratory strains and 9 strains of organisms recently isolated from urinary tract infections indicates that their activities are not widely different.

In contrast to the results obtained with streptomycin and sulfathiazole, resistance to mandelamine either did not appear, or if it did, it appeared slowly and to a questionable degree in 3 organisms, while in 3 other organisms, resistance did not appear at all.

Organisms rendered resistant to sulfathiazole or streptomycin remain susceptible to mandelamine.

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3 Scudi, J. V., *Am. J. Med. Sciences*, 1946, 211, 615.

16006 P

Differences During Dicoumarol Therapy in the Quick and Russell Viper Venom Methods for Prothrombin Determination.[*]

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We recently observed a patient with thrombophlebitis and pulmonary emboli who died in a state of hemorrhagic diathesis during dicoumarol therapy. An average daily dose

of 240 mg of dicoumarol administered for 14 days had failed to decrease the prothrombin to the desired therapeutic level as measured by the Russell viper venom modification[1,2] of Quick's method.[3] Subsequently the methods

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1 Fullerton, H. W., *Lancet*, 1940, 2, 195.

TABLE I.

Patient			Other diagnosis	Arrhythmia by EKG	Preliminary EEG	Medication for conversion to normal rhythm	EEG with normal rhythm	EKG with normal rhythm
No.	Sex	Age						
1.	♂	32	None	Paroxysmal auricular fibrillation-flutter	Abnormal Much fast 12-20 C/S	Quinidine	Normal	Normal
1a.	♂	32	None (Readmission)	Same	Same	,,	,,	,,
2.	♂	50	None	Paroxysmal auricular flutter	,,	,,	,,	,,
3.	♂	50	,,	Paroxysmal auricular fibrillation-flutter	None taken	,,	,,	,,
4.	♀	58	,,	History of parox. tachycardia, 30 years	—	—	Abnormal 4-9 C/S, High ampl. Alpha	Questionable evidence of myocardial damage
5.	♂	50	,,	History of parox. auric. fibrillation-flutter, 12 years	—	—	Borderline, Mixed frequency 8-22 C/S	Normal
6.	♂	50	Arteriosclerosis; cardiac hypertrophy	Auricular fibrillation	Normal	None attempted	—	—
7.	♂	63	Buerger's disease; amputations of 4 extremities	Extrasystoles, multiple foci. (Evidence of old myocardial infarction.)	,,	,,	—	—
8.	♂	55	History of rheumatic fever	Auricular fibrillation	,,	Quinidine	Normal	—

clinical hemorrhagic tendencies. The levels of prothrombin as determined by the Russell viper venom modification of Quick's method cannot at all times be correlated with the clinical state of the patient and the dicoumarol therapy. The control of dicoumarol therapy by the Russell viper venom method for pro- thrombin determination is a dangerous procedure. The results may be interpreted as being in a safe therapeutic level of prothrombin when actually the patient may be in a critical potential or actual hemorrhagic condition, this state being determined both by the Quick method and clinical observations.

16007

Electrocardiogram-Electroencephalogram Relations in Cardiac Arrhythmias.

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The initiating relationship of the brain, and in particular of the hypothalamus, to cardiac arrhythmias has been established on both experimental[1-4] and clinical[5-10] grounds. Although a proportion and probably a majority[11,12] of the arrhythmias may find their prime origin in intracardiac derangements, numerous cases of various cardiac arrhythmias are on record[13,14,15] in which the heart is normal. This study contains electroencephalographic data in cases of arrhythmic patients.

The electroencephalograms (EEG) were taken using the hypothalamic lead. Confirmatory electrocardiograms (EKG) were taken in each instance, except one, prior to the EEG. The EEG classification of Gibbs was used in determination of normalcy.[†] The results are summarized in the table, and tracings in Case 1-1a are reproduced in Fig. 1.

Interpretation. These cases may be divided

* Published with permission of the Chief Medical Director, Department of Medicine and Surgery, Veteran's Administration, who assumes no responsibility for opinions expressed or conclusions drawn by the author.

1 Beattie, J., Brow, G. R., and Long, C. N. H., *Res. Publ. Assn. Nerv. Ment. Dis.*, 1930, 9, 249, 295.

2 Allen, W., *Am. J. Physiol.*, 1931, 98, 344.

3 Dikshit, B. B., *J. Physiol.*, 1934, 81, 382.

4 Korth, C., Marx, H., and Weinberg, S. J., *Arch. f. Exp. Path.*, 1937, 185, 42.

5 Penfield, W. G., *Arch. Neurol. Psychiatr.*, 1929, 22, 358.

6 Bernuth, F., and Steinen, R. D., *Z. Kinderh.*, 1930, 48, 687.

7 Lucke, H., *Deutsch. Arch. f. Klin. Med.*, 1937, 180, 40.

8 Grabe, E., *Z. Ges. Neurol. n. Psychiat.*, 1930, 128, 615.

9 Ask-Upmark, E., *The Carotid Sinus*, Lund, 1935, 339.

10 Korth, C., *Ann. Int. Med.*, 1937, 11, 492.

11 Parkinson, J., and Bedford, D. E., *Quart. J. Med.*, 1927, 21, 21.

12 Parkinson, J., and Campbell, M., *Quart. J. Med.*, 1928, 22, 281.

13 Orgain, C., Wolff, H. G., and White, P., *Arch. Int. Med.*, 1937, 26, 769.

14 Stein, M. H., and Driscoll, R. E., *Ann. Int. Med.*, 1947, 26, 769.

15 Clark, R. J., *N. Eng. J. M.*, 1938, 219, 389.

† The writer wishes to acknowledge the electroencephalographic interpretations kindly furnished by David R. Talbot.

A

B
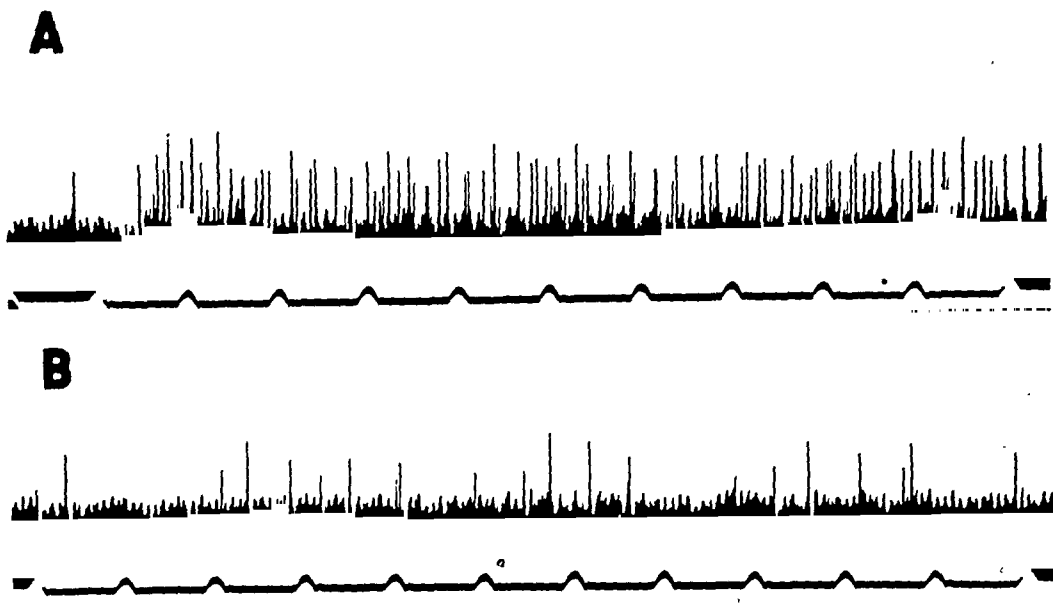
FIG. 1.
Impulses in the sciatic nerve of a frog, initiated by compression of a 5 mm length of nerve with a 40 g weight. Record A shows the impulses discharged when the weight was first suddenly applied, record B after the same weight had been kept on the nerve continuously for 10 minutes. Timer indicates ⅕ seconds.
A few larger and briefer impulses appeared during the first ⅕ second after application of the weight, as described in the text, but their oscillograph tracings were too faint for photographic reproduction.

nerve emphasize that stimulation ordinarily occurs only on the make and break of a circuit, whereas constant currents do not stimulate at all. It is obvious, therefore, that there is a discrepancy between the clinical and experimental views. This may mean that the effects observed clinically are more than the effects of simple pressure stimulation or that the classical notions of stimulation have tended to minimize certain properties of nerve which might allow for repetitive response to steady, unchanging stimuli. Recent experiments have indeed shown that under suitable conditions constant electric currents may evoke a repetitive response (Erlanger and Blair,[1] and Rosenblueth[2]). Moreover, very long sustained activity can be caused by steady chemical stimulation (Brink and Bronk,[3] Lehman[4]).

The experiments described in the present paper were undertaken to determine whether steady pressures applied to an isolated nerve preparation can similarly give rise to a continuous series of nerve impulses.

Pressure transients, that is, sudden, noninjurious increases or decreases in pressure, have been shown to initiate impulses in the sciatic nerve of the frog (Blair,[5] Schmitz and Schaefer[6]) whereas steady pressure of sufficient intensity will block conduction (Gasser[7]). It is known also that, if the nerve is subjected to an increased hydro-static pressure throughout its entire length, its excitability will be modified (Grundfest[8]). To the writers' knowledge, however, there has been no laboratory demonstration that the application of

[1] Erlanger, J., and Blair, E. A., *Am. J. Physiol.*, 1936, **114**, 328.

[2] Rosenblueth, A., *Am. J. Physiol.*, 1941, **132**, 99.

[3] Brink, F., Bronk, D. W., and Larrabee, M. G., *Ann. N. Y. Acad. Sci.*, 1946, **47**, 457.

[4] Lehman, J. E., *Am. J. Physiol.*, 1937, **118**, 613.

[5] Blair, H. A., *Am. J. Physiology*, 1935, **114**, 586.

[6] Schmitz, W., and Schaefer, H., *Arch. f. d. ges. Physiol.*, 1933, **232**, 7.

[7] Gasser, H. S., *Assn. for Research in Nervous and Mental Diseases*, 1935, ch. 2, p. 35.

[8] Grundfest, H., *Cold Spring Harbor Symposium on Quantitative Biology*, 1936, **14**, 179.
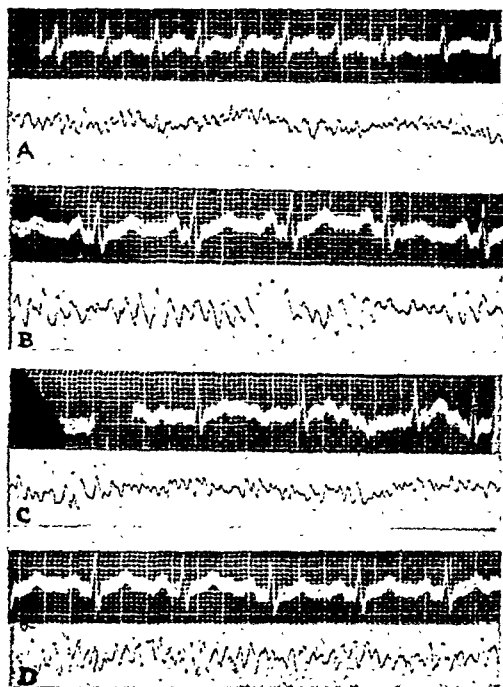
FIG. 1.

Electroencephalograms and electrocardiograms in a case of paroxysmal auricular fibrillation-flutter.
A. 3-20-47.   C. 6-20-47 (Readmission).
B. and D. After conversion to normal cardiac and encephalic rhythm, using quinidine.
B. 5-8-47.   D. 7-16-47.
EKG—Lead II.   EEG—Left.

into 2 general groups: Those in which there is EKG history and physical evidence of myocardial damage, and those without such evidence. Cases 1, 2, 3, 4, and 5 fall within the second category. Reverse cerebral excitation is not supported by findings in cases 6, 7 and 8. EEG abnormality in a case with a history of long-standing paroxysmal arrhythmia without definite evidence of myocardial damage (Cases 4, 5) may or may not be pertinent to the cardiac arrhythmia. Experimental[4] and clinical[13] demonstration of the variety of possible neurogenic cardiac arrhythmias does not make feasible any separation at this time on the basis of type of arrhythmia. Factors not associated with the arrhythmia may cause an abnormal EEG and therefore the indication that an abnormal EEG may accompany a central excitation causing the cardiac arrhythmia is gained only from Cases 1-1a, and 2. Even here it will be necessary to investigate the possibility of cerebral circulatory insufficiency secondary to the cardiac arrhythmia.

Summary. Evidence is presented to indicate that electroencephalographic abnormality coincident with neurogenic cardiac arrhythmia may disappear with restoration of normal cardiac rhythm.

16008 P

Pressure Stimulation of Peripheral Nerves.

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Clinical experience suggests that pressure upon peripheral nerves or nerve roots is accompanied by pain, as well as by alterations in sensation and other neurological changes. The radicular pains caused by neoplasms of the spinal canal, the root pains arising from herniations of the intervertebral discs and the pain caused by pressure on the brachial plexus of cervical ribs or thickened scalenus muscles are common examples. Pain occurs early and persists throughout the course of these conditions and has been attributed to the stimulating effect of the steady pressure against the nerve.

On the other hand, experimental physiological studies have repeatedly demonstrated that nerve fibers are relatively inexcitable to steady or even slowly changing stimuli. The classical studies on electrical stimulation of

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16009

Persistence of Streptomycin Resistance During Subcultures in Streptomycin Free Media.[*]

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Previous studies on the persistence of streptomycin resistance in bacteria have yielded variable results.[1-7] These studies, most of which were done with resistant variants that appeared following the growth of the organisms in culture media containing streptomycin, have indicated that the resistance of organisms usually remains essentially unchanged after prolonged storage in, or repeated transfers through streptomycin-free media.[1,6,7] In many instances, however, a diminution in the degree of resistance was demonstrated after varying numbers of transfers.[2-5] On the other hand, an apparent increase in resistance has also been observed in occasional strains.[5] Since, in all probability, these studies were concerned with bacterial populations of varying sensitivity or resistance, the results observed necessarily reflected only the most resistant strains which could multiply in the streptomycin-containing media used in the tests for sensitivity.

The purpose of the present study was to examine a group of resistant strains of various organisms, some obtained directly from patients under treatment with streptomycin and others derived *in vitro* after growth in streptomycin containing media, with a view to determining whether any loss of resistance took place in the course of 100 serial transfers through streptomycin free broth. In this study due cognizance was taken of the possibility that the final cultures might contain mixtures of organisms of varying sensitivity or resistance.

Experimental. The 13 strains selected for this study and the sources of these strains are listed in Table I. All were highly resistant to streptomycin as determined by a serial dilution method in broth.[8] Two of them (Nos. 6 and 10) were inhibited by 50,000 μg/ml but not by 10,000 μg/ml and the others were not inhibited by 50,000 μg/ml, the highest concentration used in these studies. Five of the strains (Nos. 1, 4, 7, 9 and 12) had been isolated several months earlier from patients under treatment with streptomycin; 7 (Nos. 2, 5, 6, 8, 10, 11, and 13) were resistant strains derived from originally sensitive organisms by successive passage through media containing increasing concentrations of streptomycin, and strain No. 3 was isolated only a few days before the present study began and was derived from the same parent strain as No. 2 during the course of a single 24-hour exposure to streptomycin. All of the strains except the latter one had been stored on heart infusion agar slants for 4 months at 40°C.

Each of the strains was checked for purity by first streaking on agar plates and selecting single colonies which were then transferred to brain heart infusion broth (Difco) at pH 7.4 and tested for streptomycin sensitivity. These broth cultures represented the first of the projected 100 transfers. Subsequent transfers

1 Miller, C. P., and Bohnhoff, M., *J. A. M. A.*, 1946, 130, 485.

2 Alexander, H. E., and Leidy, G., *Science*, 1946, 104, 101.

3 Graessle, O. E., and Frost, B. M., Proc. Soc. Exp. Biol. and Med., 1946, 63, 171.

4 Klein, M., and Kimmelman, L. J., *J. Bact.*, 1946, 52, 471.

5 Chandler, C. A., and Schoenbach, E. B., Proc. Soc. Exp. Biol. and Med., 1947, 64, 208.

6 Alexander, H. E., and Leidy, G., *J. Exp. Med.*, 1947, 85, 607.

7 Murray, R., Killham, L., Wilcox, C., and Finland, M., Proc. Soc. Exp. Biol. and Med., 1946, 63, 470.

8 Finland, M., Murray, R., Harris, H. W., Kilham, L., and Meads, M., *J. A. M. A.*, 1946, 132, 16.

steady pressure to a localized portion of a peripheral nerve will produce a steady discharge of impulses. The purpose of this paper is to report such observations on the sciatic nerve of the frog by means of an amplifier and oscillographic recording.

Methods. The sciatic nerve was removed from the leg of a frog and placed in a moist chamber so that the large proximal portion of the nerve rested on a waxed ebonite platform. A second ebonite block fitted over this platform and, when in position, pressed directly on a 5 mm length of the nerve. Pressures were graded by loading the block with various weights. Nerve impulses initiated by this pressure were recorded as action potentials in the smaller distal branches of the nerve.

Results. When weights of from 30-50 g were dropped on the nerve from a height of 2 mm, two types of response were elicited. At the moment of contact a transitory burst of fast, large action potentials was recorded. At the same time there was a discharge of slower and smaller impulses, which continued as long as the pressure was applied (Fig. 1). During this period the asynchronous discharge of the nerve showed a diminution in frequency analogous to "adaptation". Some fibres would "adapt" completely after a few minutes, *i.e.*, cease to respond, whereas others continued to discharge for as long as 15 minutes. Observations were terminated at the end of this period, at which time the activity had reached a fairly constant level. Gradual application of the weight elicited a discharge of the slower impulses only. When the response had at-

tained a fairly constant level some minutes after application of the stimulus, further increase of pressure increased the frequency of discharge and occasionally brought in additional fibers. Withdrawal of the weight was followed by a gradual cessation of the response.

In another series of experiments the sciatic nerve was freed by dissection in the thigh region of the frog's leg, leaving the nerve in functional continuity with the spinal cord. The spinal canal was opened to expose the sensory and motor roots which were then cut close to the cord and placed in turn on the recording electrodes. The dissected peripheral end of the nerve was arranged for pressure stimulation as in the above experiments. With this arrangement it was possible to determine whether the impulses initiated by pressure were traveling in motor or sensory fibers. A repetition of the foregoing procedures showed that the prolonged response to pressure occurred only in the dorsal roots, *i.e.* sensory fibers. The sole response from the motor roots was the transitory burst of fast impulses when the stimulus was applied. The fact that later activity was restricted to sensory fibers may depend on the presence of small as well as large fibers in this category, while the motor nerves consist almost exclusively of ·large diameter fibers. The prolonged phase of the pressure response occurs in fibers of small diameter as indicated by the relatively small amplitude and relatively long duration of the individual action potentials.

TABLE II.

Number of Colonies Grown from 0.1 ml of a 10-⁶ Dilution of the 100th Broth Transfers Seeded in Agar Containing Graded Concentrations of Streptomycin.

Strain	Final concentration of streptomycin, μg/ml						
	10,000	1,000	316	100	31.6	10	0
1*	200	202		187		194	199
2	560	585		552		586	592
3	345	340		390		390	357
4	352	347		368		442	370
5	225	270		219		240	234
6	470	480		478		500	476
7	245	246		267		280	250
8	290	289		305		280	300
9	215	228		209		247	241
10*	0	0	0	47	138	139	120
11*	0	0	0	0	98	170	179
12	290	300		300		260	323
13	280	285		260		304	290

* Average count of 4 plates for each of these strains.

tomycin. Colony counts were made after incubation for 48 hours.

The results are shown in Table II. Striking differences in the counts were noted in 2 cultures: No. 11, the strain of *Ps. aeruginosa* that was already noted to have become reduced in resistance by the serial dilution method in broth and No. 10, a strain of *E. coli*. These 2 strains produced no colonies in concentrations of streptomycin greater than 31.6 and 100 μg/ml, respectively. The remaining 11 strains showed no significant difference between the counts obtained in the streptomycin-free plates and in the plates containing streptomycin in concentrations up to 10,000 μg/ml.

It was now of some interest to determine, if possible, when in the course of the 100 transfers the more sensitive variants made their appearance and became detectable in appreciable numbers. It was also desirable to determine whether larger inocula would bring out a greater heterogeneity within the cultures of the 2 strains that had apparently become more sensitive. For these purposes, the cultures of strains 10 and 11 that had been stored on agar after the 1st, 26th, 57th and 75th transfer were subcultures in broth so that they now represented the 3d, 28th, 59th and 79th transfer, respectively. The previous experiment was now repeated with each of these subcultures, using the same inoculum, namely, 0.1 ml of a 10-⁶ dilution. In addition, the same procedure was carried

out at the same time with an inoculum of 0.1 ml of the same cultures undiluted—that is, a million times as many organisms were seeded in the same manner.

The results are shown in Table III. In the case of Strain 11 there was a sufficient difference in the number of colonies that developed from both the large and the small inoculum to indicate that an appreciable number of more sensitive cells had already appeared after 28 transfers, while there can be no doubt that this was the case after 59 transfers. This appears more clearly from the results obtained with the smaller inoculum. With Strain 10 there was similar evidence of the presence of cells of decreased resistance (increased sensitivity) at the 59th transfer.

Discussion. Of the 13 cultures studied, 11 showed no diminution in streptomycin resistance, by the methods used, during the course of 100 transfers in streptomycin free broth. In the case of 2 strains, however, the evidence indicates that organisms appeared after serial subcultures which were more sensitive to streptomycin than those of the starting cultures. The strain of *Ps. aeruginosa*, No. 11, which had originally become resistant *in vitro*, showed no growth in a concentration of 1000 μg of streptomycin per ml when tested after 100 transfers by the serial dilution method in broth. However, when a smaller inoculum was seeded in plates of agar containing graded concentrations of streptomycin (in this in-

TABLE I.
Streptomycin Sensitivities of Resistant Strains After Varying Numbers of Subcultures in Broth.

No.	Organism	Patient	Source*	1	26	57	75	100
	Strain			Number of transfers				
1	*A. aerogenes*	E.F.	a	>50†	>50	>50	>50	>50
2	,,	,,	b	,,	,,	,,	,,	,,
3	,,	,,	c	,,	,,	,,	,,	,,
4	,,	A.P.	a	,,	,,	,,	,,	,,
5	,,	,,	b	,,	,,	,,	,,	,,
6	*K. pneumoniæ*	C.M.	b	50	,,	,,	,,	,,
7	,, (atypical)	C.H.	a	>50	,,	,,	,,	,,
8	,, ,,	,,	b	,,	,,	,,	,,	,,
9	*E. coli*	M.K.	a	,,	,,	,,	,,	,,
10	,,	,,	b	50	50	,,	50	50
11	*Ps. aeruginosa*	C.M	b	>50	>50	,,	5	1
12	Paracolon bacillus	W.T.	a	,,	,,	,,	>50	>50
13	,, ,,	,,	b	,,	,,	,,	,,	,,

* a = Isolated from patients during streptomycin treatment.[8]
   b = Derived from sensitive strain after repeated subculture in increasing concentrations of streptomycin.[7]
   c = Derived from same sensitive strain as No. 2 after exposure to streptomycin for 24 hours.
   † Concentration of streptomycin, mg/ml, required for complete inhibition of growth in brain heart infusion broth, pH 7.4, with an inoculum of 10⁻⁴ ml.

were then made from the broth cultures by means of a 2 mm platinum loop. The culture tubes each contained approximately 5 ml of the brain heart infusion broth and were incubated for a period of 24 hours prior to use. The transfers were made at daily intervals at first but, as the organisms became adapted to growth on the medium, it was possible to make the transfers more frequently and as many as 3 could be made in the course of 24 hours after the 70th transfer. The cultures were examined at different stages of the experiment by streaking on heart infusion agar and on eosin-methylene blue plates in order to detect possible contaminants. All cultures remained free of contaminating organisms during the course of the experiment.

The 1st, 26th, 57th and 100th transfers were tested for streptomycin resistance by the serial dilution method in broth and the results are shown in Table I. Sub-cultures were also made at these times on agar slants and stored at 4°C for reference. All but 1 of the 13 cultures tested appeared to retain their initial streptomycin resistance through 100 transfers in streptomycin free broth. No. 11, a strain of *Pseudomonas aeruginosa* initially resistant to over 50,000 µg/ml was inhibited by 5000 µg/ml after 75 transfers and by 1000 µg/ml after 100 transfers.

Although 12 of the strains had apparently remained resistant throughout the experiment, it was not possible to say that there were not, in fact, some susceptible cells present which were masked by the more resistant ones under the conditions of the sensitivity test. If such were the case, the occurrence of the more susceptible cells might be detected by doing bacterial counts of a series of agar plates containing graded concentrations of streptomycin and seeded with the same number of organisms. This was attempted with each of the organisms using the broth culture of the 100th transfer and streptomycin concentrations of 10,000, 1000, 100, and 10 µg/ml. Additional plates containing intermediate concentrations of 316. and 31.6 µg/ml were included in the case of strains 10 and 11 which, in preliminary trials, showed marked changes.

The plates were prepared by introducing first 1.0 ml amounts of streptomycin solutions containing 10 times the desired final concentration, then 0.1 ml of a 10⁻⁶ dilution of the cultures, which experience had shown would yield about 200-300 colonies in the absence of streptomycin, and, finally, 8.9 ml of melted agar. Care was taken to avoid mixing the organisms with the streptomycin before the agar was added. Control plates were poured with the same inoculum of organisms and no strep-

subcultures in broth. Evidence was obtained which indicated that appreciable numbers of streptomycin sensitive cells appeared in 2 of these strains: in one instance after 28 subcultures and in the other after 59 transfers. The 11 remaining strains appeared to retain their resistance.

16010

Circulating Antibodies in Vitamin-Deficiency States: I. Pyridoxin, Riboflavin, and Pantothenic Acid Deficiencies.*

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(Introduced by R. R. Mellon.)

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In the study of nutrition any measure of animal performance can be utilized for the evaluation of diets. Antibody production, the normal physiological response to an antigenic stimulus, might be expected to provide a measure of the nutritional status of an experimental animal. Inasmuch as relatively few conclusive studies of this type are reported in the literature, the ability to form antibodies seems a worthy supplement to other measures of the role of various dietary factors.

Few studies have appeared concerning the effect of specific vitamin B deficiencies upon antibody production in the rat. Ruchman[1] reported that neither riboflavin nor thiamin deficiencies affected antibody production. More recently, Stoerk and Eisen[2] found reduced serum antibody concentrations in pyridoxin deficiency. Stoerk, Eisen, and John[3] have reported normal serum antibody concentration in thiamin, riboflavin, and pantothenic acid-deficient rats. Washed sheep erythrocytes were employed as the antigen in these experiments.

The present report presents data concerning the effect of pyridoxin, pantothenic acid, and riboflavin deficiencies upon antibody production by the rat in response to human red blood cells as antigenic stimulus.

Experimental. Two series of experiments differing only in the immunization procedure were conducted. Male weanling albino rats of the Sprague-Dawley strain were distributed into groups of litter mates as indicated in Table I. The animals were housed individually in wide-mesh screen-bottom cages and weighed weekly. With the exception of the group which received a colony diet (Arcady Farms) *ad libitum*, the animals were placed on a dietary regimen which consisted of a basal diet and additional vitamins in the form of a daily pill. The basal diet had the following percentage composition: sucrose, 56.76; Labco "vitamin-free" casein, 25.00; salts,[4] 4.00; cod liver oil, 2.00; hydrogenated vegetable oil, 10.00; corn oil, 2.00; choline chloride, 0.20; p-aminobenzoic acid, 0.01; i-inositol, 0.03; and 2-methyl-1, 4-naphthoquinone, 0.001. Each of the pills given to the two control groups supplied the following vitamins: thiamin, 40 γ; riboflavin, 60 γ; calcium pantothenate, 200 γ; pyridoxin, 50 γ; biotin, 1 γ; folic acid, 1 γ; and nicotinic acid, 100 γ. For the pyridoxin, riboflavin, and pantothenic acid-deficient groups, the appropriate vitamin was omitted from the

1 Ruchman, I., J. Immunol., 1946, 53, 51.

2 Stoerk, H. C., and Eisen, H. N., Proc. Soc. Exp. Biol. and Med., 1946, 62, 88.

3 Stoerk, H. C., Eisen, H. N., and John, H. M., J. Exp. Med., 1947, 85, 365.

4 Jones, J. H., and Foster, C., J. Nutrition, 1942, 24, 245.

TABLE III.

Numbers of Colonies Grown in Agar Containing Graded Concentrations of Streptomycin and Seeded with 0.1 ml Amounts of Undiluted and of 10⁻⁶ Dilution of Cultures of Resistant Organisms After Varying Numbers of Transfers in Streptomycin-free Broth.

Strain	No. of transfers	Inoculum: 0.1 ml of undiluted culture							Inoculum: 0.1 ml of 10⁻⁶ dilution of culture						
		Conc. of streptomycin, μg per ml													
		10,000	3,160	1,000	316	100	31.6	10	10,000	1,000	316	100	31.6	10	0
10	3	+++	+++	+++	+++	+++	+++	+++	50	62	55	63	70	75	63
	28	+++*	+++	+++	+++	+++	+++	+++	89	105	100	88	104	92	100
	50	+++	+++	+++	+++	+++	+++	+++	0	90	85	77	83	83	81
	77	3	40	64	4,260	+++	+++	+++	0	0	0	38	130	129	137
	100	32	45	758	3,000	+++	+++	+++	0	0	0	47	138	139	120
11	3	+++	+++	+++	+	+	+++	+++	200	230	238	220	235	218	226
	28	590	600	800	850	860	+++	+++	30	230	400	754	680	700	740
	59	154	211	260	267	690	+++	+++	0	0	0	5	150	300	342
	77	48	67	57	68	102	+	+	0	0	0	0	103	200	226
	100	34	54	65	300	332	+	+	0	0	0	0	98	170	179

* +, ++, +++ indicate increasing numbers of colonies beyond the number that could be counted.

stance about 179 organisms per 10 ml of agar) 98 colonies developed in the plate containing streptomycin in a concentration of *31.6 μg/ml* and *none grew in the plates containing 100 μg or more per ml*. On the other hand, when a much larger inoculum was used, namely 0.1 ml of the undiluted culture containing 179 million organisms, 32 colonies were counted in the plate containing 10,000 μg of streptomycin per ml and larger numbers of colonies developed in the plates which contained lower concentrations of the antibiotic.

The strain of *E. coli*, No. 10, was originally not quite as resistant as the other strains in that it was completely inhibited by 50,000 μg/ml. The serial dilution method in broth *did not demonstrate any diminution in resistance* of this strain during 100 transfers but the agar pour plate method, utilizing a small inoculum and serial concentrations of streptomycin, gave a picture similar to that found with Strain 11.

In interpreting the sensitivity tests, it *should be borne in mind that the inoculum* used in the serial dilution method in broth, as employed here, contains 0.5 ml of a 10^{-4} dilution of culture, or something in the order of 100,000 organisms. The sensitivity indicated by this method will correspond to that of the most resistant cells in the inoculum, or possibly to that of the most resistant variants that emerge during the course of the test, irrespective of their numbers, provided only that they remain viable and are capable of good growth in the media within the period of observation. If the number of resistant organisms be less than 1 per 100,000 cells, their presence might well go undetected unless a sufficiently large number of tubes were used.

Both Strain 10 and Strain 11, had originally become resistant *in vitro*. No loss of resistance was noted in any of the resistant strains which had been isolated directly from patients. The total number of strains studied, however, is too small to permit any comparison of resistant strains with respect to their origin.

Summary. Each of 13 strains of gram-negative bacilli that were highly resistant to streptomycin was passed through 100 serial

INDIVIDUAL HEMAGGLUTININ TITERS
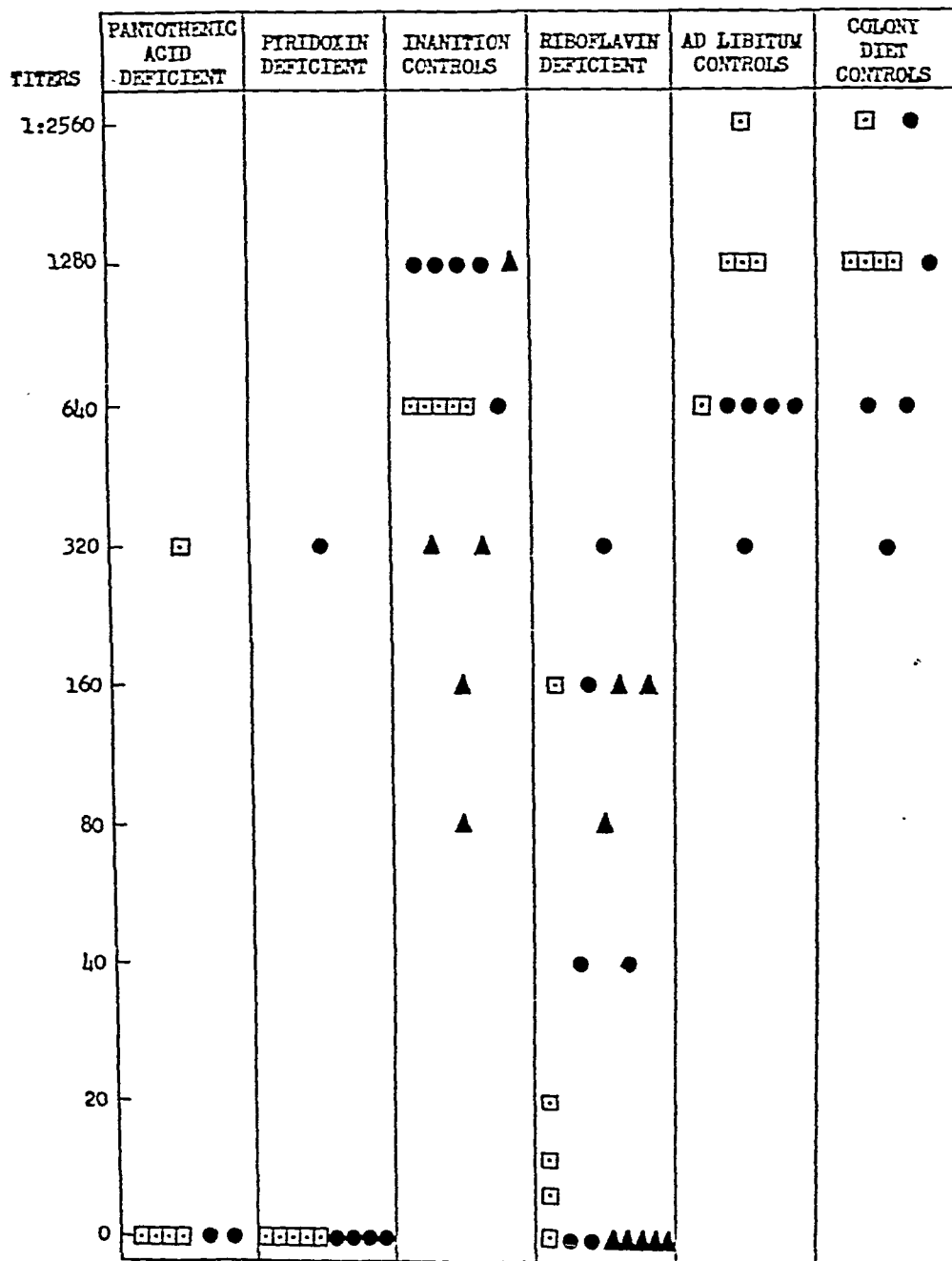
□—Series I animals.
●—Series II animals.
▲—Comparable animals in which only 3 injections (0.5, 1.0, and 1.0 ml) of cells were used as antigenic stimulus. Inanition controls were pair fed with the riboflavin-deficient group.

TABLE I.
Summary of Growth and Food Consumption Records.

Group	No. of rats	Body wt* Initial	Body wt* Final†	Daily food consumption‡
Series I.				
Pyridoxin-deficient	5	39	129	5.7
Riboflavin-deficient	5	39	48	
Pantothenic acid-deficient	5	41	100	
Inanition control	5	39	157	5.7
Control—ad libitum	5	40	329	
Colony diet	5	39	286	
Series II.				
Pyridoxin-deficient	5	46	134	5.1
Riboflavin-deficient	6	42	59	3.2
Pantothenic acid-deficient	2	45	91	4.0
Inanition control	5	45	161	5.1
Control—ad libitum	5	43	348	11.9
Colony diet	5	43	289	

* Group average in grams.
†At the time of bleeding.
‡ Group average in grams.

pill. The basal diet was fed *ad libitum* to all groups except the inanition controls, in which case the daily food intake of each rat was restricted to that consumed during the previous day by its litter mate in the pyridoxin-deficient group. In Series II, the food consumption of the remaining animals was also determined as shown in Table I.

After 7 weeks on experiment, the animals in the 3 vitamin-deficient groups had plateaued in weight and immunization of all animals was instituted. A 10% suspension of washed Group O, Rh positive human erythrocytes in normal saline was given intraperitoneally as antigen. In Series I an initial dosage of 0.5 ml of the red cell suspension was followed by 1 ml inoculations. In Series II, 0.5 ml was also employed for the primary injection, but the dosage for the subsequent injections was 1 ml of red blood cell suspension per 100 g of body weight except that the maximum dosage employed was 2.0 ml. Inoculations were made on alternate days until a total of 6 injections had been given. Five days after the final injection the rats were bled under ether anesthesia and the serum collected. The serums were tested for agglutinin titer within 24 hours after collection. Serial, 2-fold dilutions, beginning with a 1:5 dilution, were made with normal saline. A 0.2 ml sample of each dilution was incubated in 10 x 75 mm tubes at room temperature for one hour with 0.2 ml of a 2% saline suspension of Group O, Rh positive cells of the same origin as those used for the inoculations. At the end of the hour, the tubes were centrifuged lightly and read using a three-plus titer for the endpoint.

Results. The individual hemagglutinin titers are recorded in the scatter chart. It is evident that there was a marked diminution in the content of circulating antibodies in the pantothenic acid and pyridoxin-deficient groups. Thus, only one animal in each of these 2 groups possessed a measurable agglutinin titer. The fact that the agglutinin titers of the rats in the inanition control groups were equal to those of both the control animals which were fed *ad libitum* and the colony diet controls indicates that the lowered content of circulating antibodies in the pantothenic acid and pyridoxin-deficient groups cannot be ascribed to inanition *per se.* This is also borne out by the observation that, despite their poor nutritional state, the riboflavin-deficient rats possessed a higher content of circulating antibodies than either the pantothenic acid or pyridoxin-deficient rats. The content of circulating antibodies in the riboflavin-deficient rats was found to be intermediate between that of the controls and that of the pantothenic acid and pyridoxin-deficient rats. That the increased titer in the riboflavin-deficient group was not due to the

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Action of Acridines on Agents of the Psittacosis-Lymphogranuloma Group.[*]

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Inhibition of psittacosis infections in mice by trypaflavine (3, 6-Diamino-10-methyl acridinium chloride)[†] when both drug and
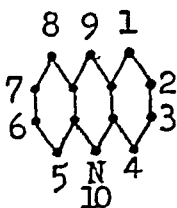
virus were given by the intraperitoneal route has been reported by Mauer.[1] Even when treatment was delayed for 7 days after inoculation of the virus, he observed some therapeutic effect. But in his experiments trypaflavine failed to inhibit cerebral or respiratory infections with psittacosis virus,[1] and Andrewes, King, and van den Ende[2] found that several of the amino acridines and atabrine were without effect on lymphogranuloma venereum virus inoculated into mice by the intracerebral or the respiratory route. Acridines with a nitro substituent at the 3-position inhibit yolk-sac infections with several species of rickettsiae,[3,4] and acriflavine is reported to be rickettsiastatic in chick embryos when the

dose is near the toxic level.[3,4] Atabrine, acriflavine, and some other aminoacridines were inactive in mice against respiratory infections with rickettsiae of murine typhus[5] and at concentrations of 1:2,000 were not lethal to this rickettsia *in vitro*. Nitroakridin 3582, or 3 - nitro - 6, 7 - dimethoxy - 9 - (2-hydroxy-3-diethylaminopropylamino) acridine, apparently has an *in vitro* virucidal action or chemoprophylactic effect on influenza virus of type B inoculated into the allantoic sac of chick embryos,[6] but other acridines were ineffective against influenza A in mice.[2] Several aminoacridines and atabrine are reported to inhibit the development of bacteriophage.[7] This paper will describe the action of some of the acridines tried by other investigators, and of a few new derivatives, on the viruses of mouse pneumonitis, cat pneumonitis, meningopneumonitis, and lymphogranuloma venereum.

Materials and Methods. The agent of mouse pneumonitis[5] and the JH strain of the virus of lymphogranuloma venereum[9] were obtained from Dr. Clara Nigg. The cat pneumonitis[10] virus was kindly sent to us by Dr. James A. Baker, and the meningopneumonitis virus,[11] of the strain Cal 10, was originally obtained from Dr. Thomas B. Turner.

Experiments in chick embryos were per-

† In designating the position of substituents in the acridine nucleus, we have used the following system of numbering throughout:

1 Mauer, G., *Zentralblatt Bakt. Abt. Orig.*, 1938, 142, 279.

2 Andrewes, C. H., King, H., and van den Ende, M., *J. Path. and Bact.*, 1943, 55, 173.

3 Smadel, J. E., Snyder, J. C., Hamilton, H. L., Fox, J. P., and Jackson, E. P., *Fed. Proc.*, 1946, II, 5, 254.

4 Smadel, J. E., Snyder, J. C., Jackson, E. B., Fox, J. P., and Hamilton, H. L., *J. Immunol.*, 1947, 57, 155.

5 Andrewes, C. H., King, H., and Walker, J., *Brit. J. Pharm. and Chemotherap.*, 1946, 1, 15.

6 Green, R. H., Rasmussen, H. F., Jr., and Smadel, J. E., *Pub. Health Rep.*, 1946, 61, 1401.

7 Fitzgerald, R. J., and Babbitt, D., *J. Immunol.*, 1946, 52, 121.

8 Nigg, C., and Eaton, M. D., *J. Exp. Med.*, 1944, 79, 497.

9 Rake, G., and Jones, H. P., *J. Exp. Med.*, 1942, 75, 497.

10 Baker, J. A., *J. Exp. Med.*, 1944, 79, 159.

11 Francis, T., Jr., and Magill, T. P., *J. Exp. Med.*, 1938, 68, 147.

higher dosage of antigen per unit of body weight is evident from the results of Series II where the dosage per 100 g of body weight was the same for all three groups. The reproducibility of these observations is demonstrated by the close agreement between the results of Series I and Series II.

Controlled hemolysin tests, using guinea pig complement, were run on serums from Series I. Hemolysin production was consistently low but followed the same pattern as the agglutinin production.

Discussion. A decrease in the content of circulating antibodies in pyridoxin deficiency was reported by Stoerk and Eisen.[2] This work is confirmed in our experiments. Our results, however, differ from those of Stoerk, Eisen, and John[3] with regard to the effects of pantothenic acid and riboflavin deficiencies. While these workers observed a normal antibody titer, we have noted a decreased titer, particularly in the pantothenic acid-deficient rats. The explanation for this difference may lie in the fact that Stoerk, Eisen, and John utilized sheep erythrocytes, whereas we employed human red cells as the antigen. The immunization procedures also differed. Since the agglutinin titers of our normal controls were considerably higher than those of Stoerk and co-workers, it is evident that our immunization procedure with human erythrocytes furnished a far stronger antigenic stimulus for agglutinin production. Sheep erythrocytes, in our hands also, have proved to be a poor stimulus for hemagglutinin production in the rat although they are effective antigens for hemolysin production. It is possible, therefore, that pantothenic acid and riboflavin do not become limiting factors for hemagglutinin production when the antigenic

stimulus is of a low order of magnitude. It is also evident from our data that riboflavin is not as critical as pantothenic acid in the production of circulating antibodies.

There is considerable basis for linking pyridoxin and riboflavin to the processes of amino acid metabolism and the role of these vitamins in antibody production may be related to these functions. To our knowledge, a similar role of pantothenic acid in protein metabolism has not been suggested. The present demonstration of the decreased antibody concentration in pantothenic acid deficiency furnishes evidence for the participation of this vitamin in protein metabolism.

As emphasized in the paper of Stoerk, Eisen, and John,[3] the decreased antibody titer in serum is not unequivocal proof for an impairment of antibody synthesis in the tissues. The possible effect of the vitamin deficiency upon the processes of antibody distribution and destruction must also be taken into account before the relationship between vitamins and antibody production can be established.

Summary. 1. Hemagglutinin production in response to inoculation with human erythrocytes has been investigated in pyridoxin, pantothenic acid, and riboflavin-deficient rats. 2. Severe impairment of antibody response was observed in the pantothenic acid and pyridoxin-deficient rats. Variable but low titers were observed in the riboflavin-deficient groups. Consistently high titers were noted in both the inanition controls and the *ad libitum*-fed animals.

TABLE II.
Effect of Acridines on Yolk Sac Infections with the Cat Pneumonitis Virus in Chick Embryos.

Drug	Dosage		Mortality ratios and degree of infection on 8th day*		% reduction of	
	mg/egg	No.	Treated egg	Control eggs	Mortality	Heavy infection
Acriflavine	.3	2	0/11 (1)†	15/16 (1)†	—100	—91
	.3	1	0/9 (2)		—100	—78
	.2	1	0/6 (4)	8/8	—100	—33
	.1	1	8/9 (1)		— 11	0
Proflavine	.3	2	4/5 (1)	6/7 (1)	— 7	0
W243	.3	3	1/14 (2)	15/18	— 91	—74
	.3	1	0/8	12/12	—100	—100
	.1	1	3/4 (1)		— 25	0
W138	.3	2	7/7	3/4	0	0
W1889	.3	1	0/8		—100	—100
	.2	1	0/9 (2)	8/8	—100	— 78
	.1	1	4/8 (1)		— 50	— 38
Atabrine	2.0	1	8/8	8/8	0	0
	.5	3	6/9 (2)	6/7	— 22	0
A1150	.2	2	8/20 (10)	17/18	— 58	— 4

* 3 to 10 LD_{50} of virus in each experiment.
†Figures in parentheses represent number of surviving eggs which were found to be heavily infected by subinoculation to mice and production of pulmonary lesions involving, on the average, over half of the lungs. Lesion scores in mice inoculated from remainder of living eggs were less than 30%.

in physiological saline which were then autoclaved at 10 lb pressure for 10 minutes. Solutions of the remaining drugs were sterilized by filtration through bacteria-retaining fritted glass discs. When used in mice the solutions or suspensions were not sterilized. In the table the lethal dose is given as the least amount which killed more than half of the mice or chick embryos. The delayed lethal dose for mice is given as the daily dose in milligrams injected intraperitoneally on 4 successive days. In chick embryos, 2 doses separated by an interval of 48 hours were inoculated into the yolk sac.

Results in chick embryos infected with the virus of cat pneumonitis. Seven acridine derivatives were tested in chick embryos against the virus of cat pneumonitis as shown in Table II. The results are evaluated in terms of mortality of the embryos by the 8th day after inoculation, at which time most of the controls were dead. In the 4th and 5th columns the figures in parentheses represent the number of additional eggs in each group which survived with heavy infection, as determined

by the mouse test. These probably would have died before the time of hatching. The remainder of the surviving chick embryos had little residual virus at 8 days or none detectable by subinoculation of mice.

The last 2 columns of the table show the percentage reduction of mortality in the treated eggs as compared with the controls, and the percentage reduction in the incidence of heavy infection. The formula 100 (-1+T/C) is used; T represents the percent mortality in the treated eggs, and C the percent mortality in the controls. For estimating the percentage reduction in heavy infections, the figures in parentheses (columns 4 and 5) were added to the numerator of each fraction and the calculations were again carried out with the formula given above. Reductions of 40% or over are italicized, and are considered to be significant.

Acriflavine and the 2 nitroacridines, W243 and W1889, were effective in single doses of 0.2 to 0.3 mg, or ⅓ to ⅕ of the lethal dose for chick embryos. With the possible exception of W1889, no appreciable effect was

formed by inoculating dilutions containing between 3 and 10 LD_{50} of egg-adapted virus, as determined by previous titration, into the yolk sacs of 6-day-old embryos and giving the drug by the same route 2 hours later. In certain experiments the injections of drug were repeated 48 and 96 hours after inoculation of the virus. Control eggs received an equivalent amount of physiological saline solution. The specificity of the deaths and the degree of viral multiplication in the yolk sacs of surviving embryos sacrificed on the 8th day were determined by subinoculation of mice by the intranasal route with yolk sac suspension from individual treated and control eggs. When the yolk sac contained little residual virus, the mice developed pulmonary lesions involving one-third or less of the lung tissue. Heavily infected yolk sacs killed part or all of the mice, and in the survivors sacrificed on the 12th day they were found to have produced a 3-plus or 4-plus pulmonary consolidation.

In experiments in the treatment of respiratory infections, mice were inoculated intranasally with dilutions of 10^{-3} to 10^{-4} of the respective mouse-adapted viruses in lung suspension, the infecting dose being adjusted by previous titration so that animals killed on the 4th day had pulmonary consolidation represented by a lesion score[‡] of 20 to 35%, while those killed on the 6th day had lesion scores of 50 to 60%. Drugs were given once daily by the intraperitoneal route beginning 2 hours after the inoculation of the virus.* Control mice received virus intranasally and saline intraperitoneally concurrently with the treated animals.

The chemical formula, source, and toxic dose of the 8 acridine compounds used in these studies is presented in Table I. For treatment of chick embryos, compounds W243, W138, and A1150 were ground with a small amount of starch to make suspensions

‡ The method of recording pulmonary lesion scores was originally used by Horsfall.[12] A lesion score of 100% represents death with complete pulmonary consolidation. In surviving mice, 80% represents, on the average, 4+ consolidation; 60%, 3+; 40%, 2+; and 20%, 1+.

TABLE I.

Chemical Name and Toxicity of the Acridine Derivatives.

No. or name	Chemical name	Lethal dose mice|| acute, mg	Lethal dose mice|| delayed, mg	Lethal dose¶ chick embryos, mg
Aciflavine*	3,6-Diamino-10-methyl acridinium chloride plus 3,6-Diamino acridine HCl	>1.0	4 x 0.5	1.0
Proflavine*	3,6-Diamino acridinium hydrogen sulfate	>1.0	4 x 0.5	1.0
W243†	3-Nitro-6,7-dimethoxy 9-(2-phenyl-4-diethylaminobutylamino) acridine 2 HCl	>1.0	4 x 1.0	1.0
W138†	3-Chloro-7-methoxy 9-(2-phenyl-4-diethylaminobutylamino) acridine 2 HCl	2.0	4 x 1.0	2 x 0.5
W1889†§	3-Nitro-6,7-dimethoxy 9-(2-phenyl-4-diethylaminopropylamino) acridine 2 HCl	—	4 x 1.0	2 x 1.0
W10†	3-Chloro-7-methoxy 9-(2-hydroxy-3-diethylaminopropylamino) acridine 2 HCl 2H$_2$O	5.0	4 x 1.0	2 x 0.5
Atabrine	3-Chloro-7-methoxy 9-(2-hydroxy-3-diethylaminopropylamino) acridine 2 HCl	5.0	4 x 1.0	2 x 1.0
A1150†	3-Nitro-9-(1-methyl-4-diethylaminobutylamino) acridine 2 HCl	2.5	4 x 1.5	2 x 1.0
	3-Nitro-9-aminoacridine HCl	1.0	4 x 0.5	2 x 0.5

* Purchased from National Aniline Division, Allied Chemical and Dye Co., Inc.
† Given by Dr. M. L. Tainter, Sterling-Winthrop Research Institute.
‡ Given by Dr. D. L. Tabern, Abbott Laboratories.
§ Same formula as Nitroakridin 3582. See references 3 and 6.
\|\| Weight 15-18 g.
¶ Delayed lethal dose represents amount of drug given on 4 successive days.
† Two doses given 48 hours apart.

TABLE IV.

Action of Acridines on the Viruses of Mouse Pneumonitis, Lymphogranuloma Venereum, and Meningopneumonitis in Mice and Chick Embryos.

Drug	Virus (10 LD$_{50}$)	Results in chick embryos*			Results in mice†		
		Dose,* mg	No. tested	% change	Dose, mg	No. tested	% change
Acriflavine	Mouse Pn.	0.3	16	— 35	0.2	40	+10
	LGV	,,	6	—100	,,	24	—40
	Meningopn.	,,	19	— 86	,,	24	—14
W243	Mouse Pn.	,,	20	— 16	0.4	31	+19
	LGV	0.2	13	— 54	,,	28	+ 6
	Meningopn.	,,	16	— 67	,,	27	— 6
W1889	Mouse Pn.	,,	10	— 70	0.3	24	—18
	LGV	,,	9	— 87	,,	24	—46
	Meningopn.	,,	9	— 89	,,	24	—40

* Drug given to chick embryos 2 and 48 hours after virus. Results expressed as reduction of incidence of heavy yolk sac infection in embryos harvested at 8 days when 75 to 100% of control embryos were heavily infected (see Table II).

† Mice received drug daily as in Table III. Results averaged for groups killed at 4 and 6 days, expressed as percentage reduction or increase in lesion scores of treated mice as compared with controls.

creum, and meningopneumonitis in chick embryos and mice. A summary of the results of preliminary experiments with 3 other viruses of the group is presented in Table IV. The drugs most effective against the cat pneumonitis virus, acriflavine and the nitroacridines W243 and W1889, were used in amounts and dosage schedules comparable to those listed in Tables II and III. In chick embryos, all 3 drugs definitely inhibited the growth of the viruses of lymphogranuloma venereum and meningopneumonitis, the effects being similar to those obtained with the agent of feline pneumonitis when expressed as reduction in incidence of heavy infection (see last column of Table II). Against the virus of mouse pneumonitis acriflavine gave results which were just below the level of significance and W243 showed no therapeutic activity. In other experiments not included in Table IV, retarded growth of this virus in embryos treated with acriflavine or W243 was observed when the yolk sacs were harvested at 5 days. The other nitroacridine (W1889) caused more definite inhibition of the growth of the mouse pneumonitis virus in chick embryos.

In mice the inhibition of respiratory infections with the 3 viruses mentioned above was less striking than that occurring in infections with the cat pneumonitis virus. None of the drugs produced appreciable reduction in the pulmonary consolidation resulting from the mouse pneumonitis virus. With one nitroacridine, W1889, results of borderline significance were obtained in tests against the viruses of lymphogranuloma venereum and meningopneumonitis, but the other nitroacridine, W243, had no effect on these 2 viruses in the lungs of mice. Acriflavine showed questionable activity against pulmonary infections with the virus of lymphogranuloma venereum and none against agent of meningopneumonitis.

Discussion. The results of these experiments suggest that replacement of the NO$_2$ group at the 3-position on the acridine nucleus by Cl results in loss, or marked diminution, of the inhibitory action against the virus of feline pneumonitis. Since the relatively simple compound 3-nitro-9-aminoacridine had some inhibitory action in chick embryos, it seems that the other substituents such as the 6- or 7- methoxy groups and the 9-dialkylaminoalkylamino side chain are not absolutely essential to the antiviral activity. The possible role of the side chain at the 9- position must be investigated further, because the compounds with this substituent seemed to be somewhat more active on weight basis than the simple 3-nitro-9-aminoacridine. Also, the preliminary results in Table IV suggest a slightly wider range of activity for the compound with the 9-(2-hydroxy-3-diethylaminopropylamino) substituent (W1889) as compared with the 9-(2-phenyl-4-diethylamino-

TABLE III.
Effect of Acridines on Respiratory Infection with Cat Pneumonitis Virus in Mice.

Drug	Daily drug dosage		Mice		Pulmonary lesion scores in %		%† reduction in pulmonary consolidation
	mg/mouse	No.	No.	Day killed	Treated mice*	Controls	
Acriflavine	0.2	4	19	4	21	37	—43
		5	19	6	52	57	— 9
Proflavine	,,	4	18	4	34	37	— 8
		5	19	6	57	57	0
W243	0.4	4	39	4	9.5	32	—70
		5	23	6	27	56	—52
W138	0.5	4	16	4	36	42	—14
		5	8	6	60	67	—10
W1889	0.4	4	12	4	2.5	24	—90
		5	30	6	5.0	56	—91
W10	0.5	5	22	6	44	48	— 8
A1150	0.2	4	26	4	21	23	— 9
		5	12	6	47	50	— 6
Penicillin G	7.9‡	4	18	4	8.9	32	—72

* Dilutions of virus 2×10^{-3} to 5×10^{-4} inoculated intranasally.
† $100(-1 + T/C)$.
‡ 1520 units of penicillin G per mg or 12,000 units per day given in 3 doses daily.

found with doses of 0.1 mg of these substances. In other preliminary experiments, some inhibitory effects were observed with the 3 drugs even when treatment was delayed for 24 or 48 hours after inoculation of the virus. The 3-nitro-9-aminoacridine, A1150, which differs from the others in having no side chain and no methoxy groups (see Table I) caused some reduction in the mortality, but was much less inhibitory than the other 3 drugs when given in equivalent dosage. Proflavine, in contrast to acriflavine, failed to influence the mortality of embryos infected with the cat pneumonitis virus under the conditions of these experiments. A slight inhibitory effect of proflavine on virus growth was demonstrable when the chick embryos were sacrificed 5 days after inoculation and the yolk sacs tested in mice. (The results of this test are not included in Table II.) The two acridines, W138 and atabrine, differ from W243 and W1889, respectively, by the presence of a single methoxy group and the substitution of a chlorine atom for the 3-nitro group. As may be seen in Table II, these 2 chloroacridines had no effect on the virus of cat pneumonitis in chick embryos when given in amounts considerably larger than the therapeutic dose of the corresponding nitroacridines.

Results in mice with cat pneumonitis virus.

Mice inoculated by the intranasal route with the virus of feline pneumonitis were given daily doses of the acridine compounds in amounts representing ½ to ⅔ of the toxic dose. The results are presented in Table III. The 3-nitroacridines (W243 and W1889) which were most effective in chick embryos also caused a significant reduction in the pulmonary consolidation of the mice, while the closely related 3-chloroacridines (W138 and W10) were inactive. Acriflavine, proflavine, and 3-nitro-9-aminoacridine (A1150) were about twice as toxic for mice as the other drugs and were, therefore, used in half the amount. The result with acriflavine in mice sacrificed 4 days after inoculation was of borderline significance, and the other compounds had no effect at the maximum tolerated dosage.

For comparison, a group of mice infected with the same virus received 12,000 units of penicillin per day divided into 3 doses of 4,000 units each and given at intervals of 7, 7, and 10 hours. The resulting inhibition of pulmonary consolidation in the mice was the same or slightly less than that caused by the nitroacridines. It should be noted that the acute lethal dose of the crystalline penicillin G used in these experiments was greater than 50,000 units.

Mouse pneumonitis, lymphogranuloma ven-

of typhus fever rickettsiae at least, is complete is shown by its activity both *in vitro* and *in vivo*. *In vitro* it is active as a complement fixation test antigen[1] and a precipitin test antigen,[5] and *in vivo* it is able to immunize guinea pigs against the disease[1] with the formation of antibodies demonstrable in the complement fixation test,[1] neutralization and precipitin tests.[6] By the use of the electron microscope, it has been demonstrated that the antigen probably arises from a capsule-like structure on the surface of the organism, and that it exists as a suspension of submicroscopic particles of the capsular substance.[7]

When purified suspensions of rickettsiae were prepared without the use of ether and subsequently shaken with ether[10] little soluble antigen could be demonstrated. When a normal yolk sac suspension was added to the purified rickettsiae, soluble antigen was released with ether. Further experiments are presented here to show that some component of egg yolk plays a part in facilitating the release of the soluble antigen from rickettsiae by ether treatment at room temperature.

The rickettsial suspensions used in these experiments were prepared from infected yolk sacs of chicken embryos. By differential centrifugation efforts were made to remove some of the yolk sac tissues and soluble proteins. The rickettsiae were finally suspended in a limited quantity of saline or distilled water containing 0.1% formalin. In the antigen release experiments generally 0.2 ml of the rickettsial suspension was mixed with 0.6 ml of diluent, either the universal buffer of

[2] Plotz, H., Reagan, R. L., and Wertman, K., PROC. SOC. EXP. BIOL. AND MED., 1944, 55, 173.

[3] Huebner, R. J., Stamps, P., and Armstrong, C., Pub. Health Rep., 1946, 61, 1605.

[4] Huebner, R. J., Jellison, W. L., and Pomerantz, C., Pub. Health Rep., 1946, 61, 1677.

[5] Shepard, C. C., and Topping, N. H., Nat. Inst. Health Bull., No. 183, Government Printing Office, Washington, 1945, p. 87.

[6] Shepard, C. C., Nat. Inst. Health Bull. No. 188, Government Printing Office, Washington, 1945, p. 93.

[7] Shepard, C. C., and Wyckoff, R. W. G., Pub. Health Rep., 1946, 61, 761.

TABLE I.

Effect of Normal Egg Yolk on Release of Antigen from Rickettsiae. Homologous convalescent guinea pig serum was used in each instance.

Soluble antigen from	Conc. of soluble antigen (as original yolk sac) %	pH	Diluent	Complement fixation results Dilutions of soluble antigen						Antigen control 1:4
				1:4	1:8	1:16	1:32	1:64	1:128	
R. prowazeki* Brehl strain)	25	6.14	2.5% yolk	++++	++++	++++	++	—	—	—
		6.10	Buffer only	+++	—	—	—	—	—	—
R. mooseri* (Wilmington strain)	15	6.1	2.5% yolk	++++	++++	++++	++++	++	—	—
		6.0	Buffer only	+	+++	+	—	—	—	—
R. rickettsii† (Bitter Root strain)	50	6.3	2.5% yolk	+++	++++	++++	++++	+++	trace	—
		5.6	Buffer only	+++	trace	—	—	—	—	—
R. akari‡ (Wild mouse strain)	25	6.0	2.5% yolk	+++	++++	++++	trace	—	—	—
			Buffer only	+++	++++	trace	trace	—	—	—
R. burnetii (Italian strain)	50	5.95	2.5% yolk	—	—	—	—	—	—	—
		6.14								
		6.10	Buffer only	—	—	—	—	—	—	—

* Rickettsiae were washed with salt solution pH 7.6 and distilled water according to the method of Shepard and Topping.[10]

† Rickettsiae were sedimented two times and resuspended in distilled water.

‡ Rickettsiae were sedimented once, then resuspended in saline.

butylamino) group (W243).

It remains to be seen whether acriflavine acts in the same manner as the nitroacridines. The active constituent of acriflavine seems to be the 10-methyl acridinium chloride (see Mauer[1]). Proflavine, which is the sulphuric acid salt without the 10-methyl group, was much less inhibitory for the feline pneumonitis agent in chick embryos. Its activity against other viruses of the group is under investigation.

The relative resistance of the mouse pneumonitis virus to acriflavine and to one of the nitroacridines is surprising in view of the high sensitivity of this agent to penicillin[13,14] and sulfonamides.[15] The feline pneumonitis and meningopneumonitis viruses which are, on the other hand, relatively resistant to penicillin and are not affected in mice or chick embryos by sulfonamides are quite readily inhibited in chick embryos by acriflavine and the nitroacridines. The observed differences in the chemotherapeutic spectrum for penicillin, sulfonamides, and acridines suggest interesting variations in the enzymatic or metabolic constitution of the agents of the psittacosis-lymphogranuloma group. The 3 classes of substances probably act on 3 different constituents of the viruses under consideration.

The results in mice tended to parallel those in chick embryos, but on a lower scale of activity so that less conclusive results were obtained. Effective inhibition of respiratory infections was observed only with the 2 nitroacridines against the cat pneumonitis agent, but these 2 substances were, on a weight basis, more effective than penicillin against this virus. Because of the high toxicity of acridines and their failure to show general activity a g a i n s t the psittacosis-lymphogranuloma group in mice, no claim for their therapeutic usefulness can be made at the present time.

Summary. Acriflavine; 3-nitro-6, 7-dimethoxy 9 - (2-phenyl-4-diethylaminobutyl-amino) acridine; and 3-nitro-6, 7-dimethoxy 9 - (2-hydroxy - 3-diethylaminopropylamino) acridine inhibited yolk sac infections of chick embryos with the agents of feline pneumonitis, lymphogranuloma venereum, and meningopneumonitis. The first two compounds were less active against the virus of mouse pneumonitis, but the last-named inhibited this agent in chick embryos.

Proflavine, atabrine, and drugs closely related to the above-mentioned nitroacridines, except for substitution of Cl for NO_2, had no significant inhibitory action. 3-nitro-9-amino-acridine was intermediate in its effect.

Respiratory infections in mice caused by the agent of feline pneumonitis were retarded by the two nitroacridines, but these drugs showed slight or no effect in mice against intranasal infection with the agents of mouse pneumonitis, lymphogranuloma venereum, and meningopneumonitis.

12 Horsfall, F. L., Jr., *J. Exp. Med.*, 1939, **70**, 209.

13 Meiklejohn, G., Wagner, J. C., and Beveridge, G. W., *J. Immunol.*, 1946, **54**, 1.

14 Eaton, M. D., Dozois, T. F., van Allen, A., Parish, V. L., and Schwalm, S., *J. Immunol.*, in press.

15 Eaton, M. D., and Hanford, V. L., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 63.

16012

Effect of Egg Yolk on Release of Antigen from Rickettsiae.

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The existence of a soluble antigen which is released on treatment of certain rickettsiae with ether has been well established with respect to *Rickettsia prowazeki,*[1] *R. rickettsii,*[2]

and *R. akari.*[3,4] That this antigen, in the case

1 Topping, N. H., and Shear, M. J., *Pub. Health Rep.*, 1944, **59**, 1671.

TABLE I.
Effects on Renal Function of Adrenaline, Tetraethylammonium, and "Isuprel."

Dog	Per.	C_{PAH} cc per min	C_{CR}	FF	R	B P mm Hg	Pulse	Duration min
4-9 14 kg	1-2	208	62.6	0.30	0.27	149	110	20
				Adrenaline 0.1 cc 1/1000 i.v.				
	3-4	198	63.7	0.32	0.29	152	130	20
				Et₄N 2 cc 10% i.v.				
	5-6	254	69	0.27	0.18	129	135	20
				Adrenaline 0.1 cc 1/1000 i.v.				
	7	163	51	0.31	0.39	161	100	10
	8	197	60	0.30	0.22	128	112	10
6-3 18 kg	1-2	178	46	0.26	0.24	141	98	20
				Adrenaline 0.1 cc 1/1000 i.v.				
	3-4	130	47.4	0.36	0.31	140	100	20
				Et₄N 2 cc 10% i.v.				
	5-6	145	40.4	0.28	0.27	134	130	20
				Adrenaline 0.1 cc 1/1000 i.v.				
	7-8	111	32.5	0.29	0.46	158	84	18
4-9 14 kg	1-2	149	56	0.37	0.32	149	110	20
				"Isuprel" 0.2 cc 1/1000 i.v.				
	3	144	58	0.40	0.29	150	145	10
	4	175	58	0.33	0.20	123	150	31
	5	169	40	0.24	0.29	147	120	20
				Et₄N 2 cc 10% i.v.				
	6	170	53	0.31	0.28	147	140	30
	7	129	49	0.38	0.33	140	130	20
				"Isuprel" 0.2 cc 1/1000 i.v.				
	8	68	24	0.35	0.17	83	148	15
	9	278	46	0.31	0.10	86	176	20
	10	251	46	0.34	0.19	107	172	12
3-7 12 kg	1-3	218	74	0.34	0.21	135	55	30
				"Isuprel" 0.1 cc 1/1000				
	4-5	202	61	0.30	0.19	122	80	20
				Et₄N 2 cc 10% i.v.				
	6-7	236	58	0.25	0.14	114	128	30
				"Isuprel" 0.1 cc 1/1000 i.v.				
	8	223	63	0.28	0.10	93	140	20
	9	231	68	0.29	0.20	136	112	20

Dog number and body weight are indicated in the left hand column. Per. = intervals of urine collection. C_{PAH} = plasma clearance of p-aminohippurate. C_{CR} = plasma clearance of creatinine. FF = filtration fraction (C_{CR}/C_{PAH}). R = renal resistance as sum of R_A and R_E calculated by the method of Lamport. B.P. = average of arterial pressure during the time of the observation. Pulse = average of pulse rate during time of observation. Duration of the observation is indicated in minutes.

this and similar compounds resembles that posited for sympathin I, since many of its effects contrast with those of adrenaline and with those credited to sympathin E. Since adrenaline is a renal vasoconstrictor, it seemed likely that 'Isuprel' might be a renal vasodilator. One of the purposes of this report is to describe the effect of injection of "Isuprel" on the renal circulation.

Another aspect rests on the observation of Page and Taylor[2] that the effect on arterial pressure of adrenaline and other pressor drugs is increased by pre-treatment with tetraethylammonium (Et₄N). We demonstrate below that potentiation of the action of adrenaline by pre-treatment with Et₄N is reflected in renal circulation as well as arterial pressure and (b) that the depressor, cardiac accelerator and renal vasodilator properties of "Isuprel" are similarly increased.

Procedures. Six experiments were done, 3 each with adrenaline and 'Isuprel', on trained conscious dogs. In these renal plasma clearances of p-aminohippurate and creatinine

[2] Page, I. H., and Taylor, R. D., *Science*, 1947, 105, 622.

Michaelis[8] or the same buffer containing 2.5% of normal yolk from fresh chicken eggs. To each mixture were added 1.5 ml of anaesthetic ether. The two phases were then shaken together vigorously 30 times. The aqueous layer was drawn off, freed of ether with vacuum and centrifuged at 4000 r.p.m. for one hour to sediment the rickettsiae. The supernatants were tested for antigen content by the complement fixation test[9] using 4 units of homologous convalescent guinea pig serum. The results of the tests with five species of rickettsiae are shown in the table.

The readings in the table show that in the presence of 2.5% yolk higher titers were obtained with *R. prowazeki, R. mooseri, R. akari,* and *R. rickettsii.* With *R. burneti* no soluble antigen could be detected.

When normal yolk was diluted 10 times with 0.85% sodium chloride solution and extracted with 2 volumes of ether, it was found that the water soluble constituents in the aqueous layer had the same degree of activity in releasing soluble antigen from rickettsiae as the original yolk.

Although it will be noted that some antigen was released without the addition of yolk, such release may have been due to the amount of yolk remaining with the preparation of rickettsiae, since the more carefully purified

Breinl and Wilmington suspensions showed little release except when yolk was added. It has been noted that the increase in titer obtained by adding yolk is variable unless the rickettsiae are carefully washed. Yolk sacs are known to carry more or less yolk after harvest and it is evident that the amount of adherent yolk can effect the complement fixation titer of an ether extracted antigen. For consistent results it has been found advantageous to add 5 or 10% normal egg yolk to all method I and method II antigens[11] for use in complement fixation tests.

In the preparation of Rocky Mountain spotted fever antigens by method I or II,[11] the addition of yolk is especially valuable[12] since the growth of *R. rickettsii* in yolk sacs is poor, and antigen release is frequently not marked.

Summary. The addition of as little as 2.5% egg yolk to suspensions of yolk sacs infected with the rickettsiae of epidemic and endemic typhus fever, Rocky Mountain spotted fever, and rickettsialpox (*R. prowazeki, R. mooseri, R. rickettsii,* and *R. akari*) prior to treatment with ether has resulted in antigens with enhanced titers as measured by the complement fixation test.

8 Michaelis, L., *Biochem. Z.,* 1931, **234**, 139.

9 Bengtson, I. A., *Pub. Health Rep.,* 1944, **59**, 402.

10 Shepard, C. C., and Topping, N. H., *J. Immunol.,* 1947, **55**, 97.

11 Topping, N. H., and Shepard, C. C., *Pub. Health Rep.,* 1946, **61**, 701.

12 Unpublished experiments.

16013

Renal Hemodynamic Effects of Adrenaline and "Isuprel": Potentiation of Effects of Both Drugs by Tetraethylammonium.

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Vasodepressor drugs have been prepared which are built on the chemical nucleus of sympatheticomimetic compounds. The pharmacological properties of one of these, the N-isopropyl homologue of adrenaline, called "Isuprel" (1-(3[1],4[1]-dihydroxyphenyl)-2-iso-

propylaminoethanol hydrochloride) have been described by Lands and his colleagues.[1] The suggestion has been made that the activity of

1 Lands, A. M., Nash, V. L., McCarthy, H. M., Grainger, H. R., and Dertinger, B. L., *J. Pharm. Exp. Therap.,* 1947, **80**, 110.

terial pressure and renal resistance in 2 of 3 experiments. A second injection of "Isuprel" resulted in hypotension, tachycardia and decreased renal resistance which were greater in extent and duration than the effects observed before injection of Et₄N. In the anesthetized dog, the vasodepressor effect of "Isuprel" is shown to be increased by injection of Et₄N. The augmentation is demonstrable at 5 but not at 30 minutes after injection of Et₄N. The reactive pressor effect of "Isuprel" after injection of Et₄N was observed in other experiments on anesthetized dogs.

Comment and Summary. The increased pressor responsiveness to adrenaline caused by injection of Et₄N is shown to be associated with an increase in renal vasoconstriction. The depressor response to injection of "Isuprel," the N-isopropyl homologue of adrenaline, is shown to be associated with renal vasodilation. The depressor, cardioaccelerator and renal vasodilator effects of injection of "Isuprel" are augmented by prior injection of tetraethylammonium. On the assumption that the effects of tetraethylammonium are due to blocking of autonomic ganglia,[4] we conclude that inhibition of these ganglia increases vascular responsiveness to typical vasoconstrictor and vasodilator sympatheticomimetics.

4 Acheson, G. H., and Moe, G. K., *J. Pharm. Exp. Therap.*, 1945, **84**, 189.

16014 P

Pharmacology of Dibenzyl-β-Chloroethylamine Hydrochloride (Dibenamine).*

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Recently Nickerson, G o o d m a n and Smith,[1,2,3] Raab and Humphreys,[4] Acheson and co-workers,[5] Haimovici[6] reported their experiments with dibenzyl-β-chloroethylamine hydrochloride (dibenamine).

We wished to investigate further the pharmacology of dibenamine and the mechanism of transmission of sympathetic nerve impulses.

Methods. The experiments have been carried out on dogs under chloralosane anesthesia. The blood pressure was registered from a femoral artery. In some experiments the peripheral vasomotor reactions were recorded by means of the 3-manometers-method.[7]

Results. I. *Effects of Dibenamine on blood pressure and heart rate.* Dibenamine, from 1 to 5 mg/kg, does not induce a noticeable change in blood pressure, nor any variations of heart rate. Doses of 10 to 15 mg/kg produce a slight and transient hypotension, but no direct change of heart rate. Subsequent injections of dibenamine do not produce the some hypotension. A primary, slight increase

1 Nickerson, M., and Goodman, L. S., *Fed. Proc.*, 1946, 5, 194.

2 Nickerson, M., Smith, S. M., and Goodman, L. S., *Fed. Proc.*, 1946, 5, 195.

3 Nickerson, M., and Goodman, L. S., *J. Pharm. Exp. Therap.*, 1947, 89, 167.

4 Raab, W., and Humphreys, R. J., *J. Pharm. Exp. Therap.*, 1946, 88, 268.

5 Acheson, G. H., and co-workers, *Fed. Proc.*, 1947, 6, 305.

6 Haimovici, H., Proc. Soc. Exp. Biol. and Med., 1947, 64, 486.

7 Nolf, P., *Bull. Acad. Roy. Belg.*, 1902, p. 895.

were determined. The plasma clearance of p-aminohippurate is taken as equivalent to renal plasma flow and the plasma clearance of creatinine as equivalent to the rate of glomerular filtration. Femoral arterial pressure was measured from a mercury manometer and inlying needle. Each experiment consisted of (a) 2 or 3 control periods of clearance measurement; (b) observations after intravenous infusion of small doses of either adrenalin or "Isuprel"; (c) observations after infusion of Et_4N and (d) observations after a second injection of either of the 2 sympatheticomimetics. One experiment was done in a dog anesthetized with pentobarbital. In this only "Isuprel" and Et_4N were injected.

Results. Observations in 2 experiments on conscious dogs in which adrenaline and Et_4N were injected and in 2 in which "Isuprel" and Et_4N were tested are summarized in Table
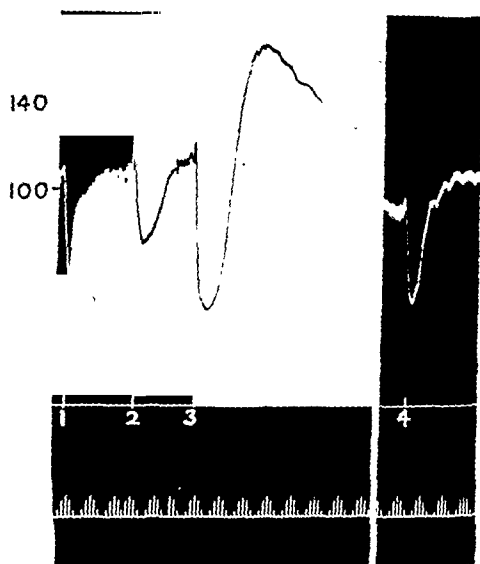
FIG. 2.

Effect on arterial pressure of injection of "Isuprel" and Et_4N in an anesthetized dog, of 10.7 kg body weight. At 1 there was injected 0.2 cc of 1/20,000 "Isuprel." At 2 the animal was given intravenously 10 mg of tetraethylammonium chloride per kg body weight. At 3 the dose of "Isuprel" was repeated. At 4, 30 minutes after injection of tetraethylammonium, the same dose of "Isuprel" was again injected.

I. The course of one experiment of each type is shown graphically in Fig. 1. Fig. 2 demonstrates the effect on arterial pressure of injections of "Isuprel" Et_4N in the anesthetized dog.

Adrenaline. The first injection of adrenaline caused a slight, transient increase of arterial pressure with some increase in renal vascular resistance as calculated by the method of Lamport.[3] Injection of Et_4N slightly decreased renal resistance and arterial pressure in 2 of the 3 experiments. A second injection of adrenaline 20 minutes after injection of Et_4N resulted in increases of arterial pressure and renal vascular resistance which were much greater than the responses to the first injection.

"Isuprel." The first injection caused transient hypotension and tachycardia and a modest decrease in renal resistance. Injection of Et_4N caused again slightly decreased ar-

FIG. 1.

Graphic summary of 2 experiments. C_{PAH} = renal plasma clearance of p-aminohippurate. C_{cr} = plasma or creatinine clearance. B. P. mm Hg = arterial pressure and is shown from repeated observations of femoral arterial pressure. R = renal resistance, and, for convenience, is shown times 100. The experiment on the left was done on dog No. 4-9, body weight 14 kg, and corresponds to the first experiment in Table I. The experiment on the right was done on dog No. 3-7 and corresponds to the last experiment in Table I.

3 Lamport, H., *J. Clin. Invest.*, 1943, 22, 461.

16015 P

Mitoses in the Livers of Rats Treated with Thiourea.

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From the Department of Experimental Pathology, The Hebrew University, and the Rothschild Hadassah University Hospital, Jerusalem, Palestine.

The purpose of the present communication is to report the remarkable effect of large doses of thiourea on the liver of rats. This effect is manifested by the appearance of numerous mitoses in the liver cells.

Material and Methods. Thirty-six young male albino rats, weighing between 70 and 100 g were used in these experiments. Four-tenths of a gram of thiourea in a 10% aqueous solution was injected by the intraperitoneal route at daily intervals. Six rats died a few hours after the first injection, 3 succumbed after the second and 8 after the third. Fifteen rats survived 3 injections and were sacrificed 2 to 12 hours after the last injection. Of the remaining 4 rats 3 were sacrificed after 4, and 1 after 7 injections.

The livers were fixed in Carnoy's fluid or in Zenker's solution. Paraffin-celloidin sections 5μ in thickness were stained with hematoxylin-eosin or with Heidenhain's hematoxylin.

Results. In the 23 rats which succumbed or which were sacrificed after 3 daily injections of thiourea, the gross appearance of the livers was normal. Microscopically, there was central congestion of the sinusoids and slight to moderate vacuolization of the liver cells in the central parts of the lobule, as well as slight alterations in the lining of the central vein. The outstanding feature was the presence of all stages of mitotic figures in the liver cells (Fig. 1). Mitoses were found in all animals receiving this treatment, but there were variations in the number of mitoses from rat to rat. In 22% of the treated rats the number of mitoses was extremely high (12 to 18 mitoses in each microscopic field):* in 26% they were numerous (6 to 12 mitoses

in each microscopic field); a moderate number of mitoses was present in 35% of the animals (1 to 6 mitoses in each microscopic field) and in 17% of the treated rats the number of mitoses was small (one mitosis being found in 1 to 3 microscopic fields).

The configuration of the mitoses was in most cases completely normal and the different phases of the mitotic processes were encountered in the usual proportions. In a number of livers abnormal mitotic figures were found in moderate or appreciable numbers. The pathological mitoses were characterized by clumping or scattering of the chromosomes. In some experiments mitosis with widely scattered chromosomes was the only type encountered. At the result of chromosomal scattering, liver cells with bizarre nuclear structures appeared at times: some loaded with minute nuclei and others with single or multiple large, lobated nuclei. The multinuclear liver cells were often exceptionally large.

In rats which died or were sacrificed within 24 to 31 hours after the first injection no mitoses were found in the liver cells. After 4 injections, *i.e.*, 72 to 96 hours after the start of the experiment, the number of mitoses was considerably lower than after 3 injections. In one rat examined after 7 injections no mitoses were found. It is thus evident that the mitotic effect is not immediate, but appears only after a certain latent period. The extensive mitotic activity of the liver seems to be limited to a time period of 48 to 72 hours after the first injection.

Comment. The occurrence of mitoses in the liver or in other visceral organs, excepting for the thyroid gland, following treatment with thiourea has not as yet been noted. In the thyroid glands of rats, receiving thiourea,

* The mitotic counts were made in microscopic fields of 0.4 mm diameter.

of blood pressure has been recorded in some experiments.

II. *Localization of the hypotensive action of dibenamine.* No noticeable changes in blood pressure have been observed after injection of dibenamine into the carotid artery (to control a possible central action), while small quantities of dibenamine injected into the peripheral circulation produced an immediate local vasodilatation.

III. *Effects of Dibenamine upon the carotid sinus vasomotor reflexes.* Dibenamine (0.5 to 15 mg/kg) does not produce a marked change of the pressor and depressor carotid sinus reflexes on the arterial blood pressure; same doses, however, reverse the pressor action of epinephrine into a vasodepressor one. Only 60 to 90 minutes after the injection of high doses of dibenamine, a depression, but not a reversal of the carotid sinus vasopressor reflexes could be observed.

IV. *Effects of Dibenamine on hypertensive substances.* Reversal of the vasopressor action of epinephrine appears after administration of at least 10 mg/kg dibenamine. But dibenamine does not suppress or reverse the epinephrine tachycardia. Small quantities of dibenamine, injected into the circulation of the leg, induce a strictly localized reversal of the vasoconstrictor effect of epinephrine.

Ten to 15 mg/kg dibenamine do not reverse the hypertensive action of nicotine in the atropinized dog. Larger quantities decrease slightly, but never reverse, the vasopressor effects of nicotine.

In atropinized dogs, with normal adrenal glands, 10 to 15 mg/kg dibenamine convert the vasopressor action of acetylcholine into a vasodilator response.

The vasoconstrictor effects of ephedrine and pituitrin are scarcely altered by dibenamine.

Ten to 15 mg/kg dibenamine convert the asphyxia hypertension into an hypotension.

V. *Effect of Dibenamine on sympathetic nerve stimulation.* In dogs, with adrenal glands, after injection of 10 to 15 mg/kg dibenamine, the hypertensive response of splanchnic nerve stimulation is converted into a vasodilatation, after an initial rise of the blood pressure.

In dibenamine-treated dogs, after adrenalectomy, the stimulation of the splanchnic nerve produces a monophasic or no rise of the blood pressure; no reversal of this neurovasopressor effect has been observed.

After injection of dibenamine, the general reflex vasoconstrictor response, induced by stimulation of the central end of the vagus, is not converted into a vasodilatation.

Summary. Experiments on dogs showed that dibenamine:

1. Induces a slight fall of arterial pressure; this hypotension depends mainly on a peripheral vasodilator action.

2. Has no direct effect upon the heart rate.

3. Produces no lasting decrease of the carotid sinus vasopressor reflexes; it never induces a reversal of these reflexes, while the same dose reverses completely the action of epinephrine on blood pressure and blood vessels.

4. Does not affect the tachycardic action of epinephrine.

5. Has no marked effect upon the hypertensive properties of nicotine, ephedrine and pituitrin.

6. Produces a reversal of the asphyxia hypertension.

7. Induces, in atropinized dogs, a reversal of the vasopressor action of acetylcholine.

8. May decrease, but does not produce a reversal of the vasomotor responses of sympathetic origin.

9. Is primarily a powerful adrenolytic, but a weak sympathicolytic agent.

16015 P

Mitoses in the Livers of Rats Treated with Thiourea.

M. Rachmilewitz, A. Rosin, and L. Doljanski.

From the Department of Experimental Pathology, The Hebrew University, and the Rothschild Hadassah University Hospital, Jerusalem, Palestine.

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Material and Methods. Thirty-six young male albino rats, weighing between 70 and 100 g were used in these experiments. Four-tenths of a gram of thiourea in a 10% aqueous solution was injected by the intraperitoneal route at daily intervals. Six rats died a few hours after the first injection, 3 succumbed after the second and 8 after the third. Fifteen rats survived 3 injections and were sacrificed 2 to 12 hours after the last injection. Of the remaining 4 rats 3 were sacrificed after 4, and 1 after 7 injections.

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FIG. 1.
Liver of rat treated with 3 doses of 0.4 g thiourea. Mitoses in liver
cells. Hematoxylin-eosin. × 850.

mitoses were described by Paschkis and associates.[1]

Mitoses in the normal liver of the adult rat are extremely rare.[2] It is well known, however, that loss of liver tissue by removal of a part of the organ or by necrosis is followed by a compensatory multiplication of liver cells. It was occasionally noted that mitoses in the liver may also occur in the absence of visible changes in the hepatic parenchyma after the administration of staphylococcus toxin,[3] ma-

cerated livers, trypaflavine[4] or trypan blue.[5,6] No satisfactory explanation has been advanced for a mitotic reaction of this kind.

The nature of the mitotic response of liver cells to thiourea is likewise not clear. The possibility may be considered that thiourea has a direct stimulating effect on the mitotic activity of the liver cells. On the other hand, occurrence of mitoses may be regarded as a secondary phenomenon, namely as a reparatory reaction following discrete liver damage. The arguments in favor or against each of these possibilities will be dealt with in the complete communication.

Summary. The occurrence of mitosis in the liver cells of rats receiving daily intraperitoneal injections of thiourea is reported. Both mitosis of normal configuration and pathological mitosis were encountered. Thiourea induced mitotic activity of the liver seemed to be at its height during the period of 48 to 72 hours after the first injection.

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5 Pfuhl, W., *Z. f. Anat. u. Entwicklungsgesch.*, 1938/9, **109**, 99.

6 Deane, H. W., *Anat. Rec.*, 1944, **88**, 245.

16016

Role of the Vagus Nerve in Experimental Cinchophen-Ulcer.

JOHN R. HILSABECK AND FREDERICK C. HILL.

From the Departments of Physiology and Experimental Surgery, The Creighton University School of Medicine.

By giving the drug cinchophen, it is possible to produce a peptic ulcer in the dog pathologically identical with that found in man,[1] and, furthermore, procedures which have benefited one have been equally helpful in the other,[2-6] and vice versa.[7,8] Cinchophen produces an ulcer in almost 100% of dogs without grossly disturbing the normal physiology of the gastro-intestinal tract,[9] and provides an ideal means for observing the effect of various proposed measures on peptic ulcer.

Numerous studies on cinchophen-ulcer have been conducted by Stalker, Bollman and Mann.[1,3,5,10] They came to the conclusion that cinchophen produced an increase in the total volume of gastric secretion and that the increase was chiefly acid.[10] Thus, it was assumed that cinchophen caused an ulcer by producing hyperacidity.

If cinchophen-ulcer is caused by an increase in the total volume of acid, it is clear that transthoracic vagotomy, by its reduction of gastric acidity,[11] should have a

marked beneficial effect on this ulcer. To verify this inference, transthoracic supradiaphragmatic vagotomy was performed in 15 dogs. These vagotomized animals were then given sufficient cinchophen to produce an ulcer over varying lengths of time.

Procedure. The vagus nerves were observed in the thorax and abdomen at autopsy in 42 dogs and were found to pursue a constant course. Operations were performed under positive intratracheal pressure and morphine-pentobarbital anesthesia. The right chest was opened in the 7th interspace and a 1.5 to 3.0 cm section removed from the anterior and posterior vagus nerves just before they pierced the diaphragm. The chest was closed by interrupted cotton sutures throughout.

Two groups of vagotomized dogs were used. The first, Group A, comprising 4 dogs, received 2 g of cinchophen 4 times a week for at least 30 days (this has been reported as giving the greatest incidence of chronic cinchophen-ulcer[3]) to allow an ulcer to form. Then, 2 g of the drug were given every day to try to perforate the ulcer if present.

The second group, Group B, comprising 11 dogs, received approximately 2 g of cinchophen 6 days a week. This method had been used previously in this laboratory to produce cinchophen-ulcer.[6,7,8]

Results. All the dogs in Group A developed ulcer. Nine dogs in Group B developed ulcer; however, in 2 of these animals the vagi had not been completely severed in the thorax: in one, the posterior branch of the right vagus was intact; in the other, the anterior vagus was intact. The 2 animals in Group B that failed to develop an ulcer received cinchophen for an insignificant period of time—2 to 9 days respectively. All dogs had excellent appetites throughout cinchophen administration until the last day or so before

[1] Stalker, L. K., Bollman, J. L., and Mann, F. C., *Arch. Surg.*, 1937, 35, 290.

[2] Reid, P. E., and Ivy, A. C., PROC. SOC. EXP. BIOL. AND MED., 1936, 34, 142.

[3] Bollman, J. L., Stalker, L. K., and Mann, F. C., *Arch. Int. Med.*, 1938, 61, 119.

[4] Winters, M., Peters, G. A., and Crook, G. W., *Am. J. Dig. Dis.*, 1939, 6, 12.

[5] Stalker, L. K., Bollman, J. L., and Mann, F. C., *Am. J. Dig. Dis. and Nutr.*, 1937, 3, 822.

[6] Andersen, A. C., and Hill, F. C., *Bull. Creighton School of Medicine*, 1942, 5, 2.

[7] Slutzky, B., Wilhelmj, C. M., and Stoner, M. E., *Am. J. Dig. Dis.*, 1941, 8, 469.

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[10] Stalker, L. K., Bollman, J. L., and Mann, F. C., *Arch. Surg.*, 1937, 34, 1172.
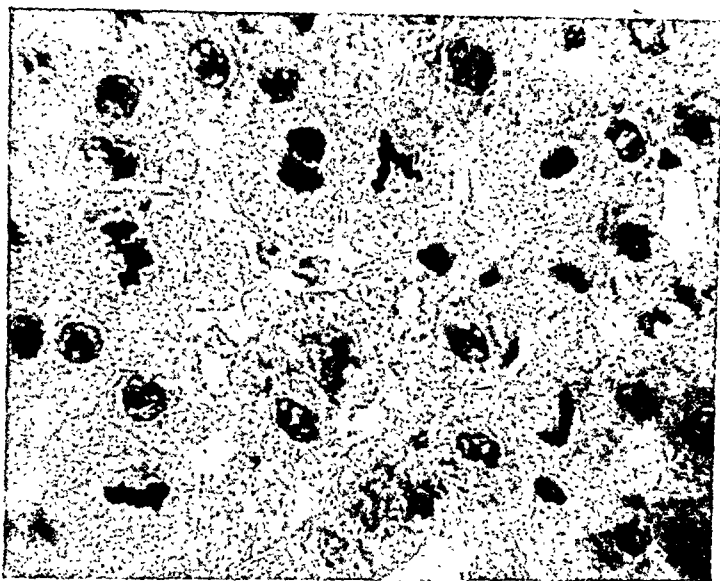
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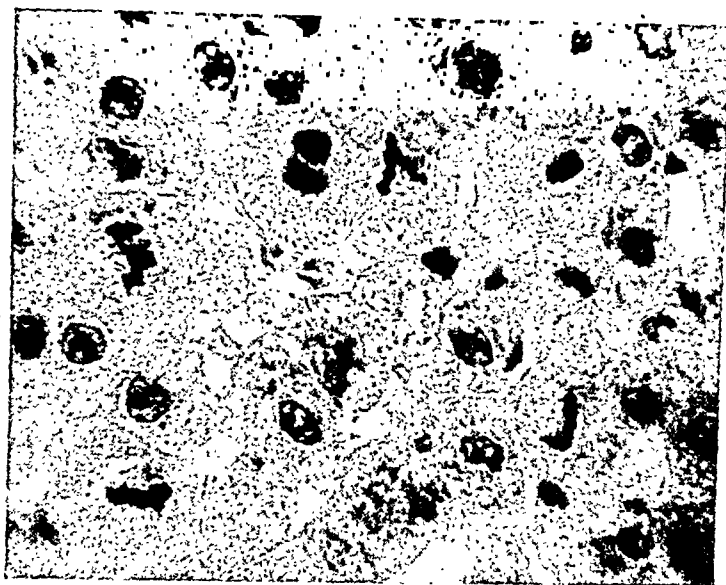[11] Hartzell, J. B., *Am. J. Physiol.*, 1929, **91**, 161.

TABLE I.
Cinchophen Administration to Vagotomized Animals.

Dog	Total grams of cinchophen	Days	Results
1*	4	2	Stomach and duodenum normal
2*	18	9	,, ,, ,, ,,
3*	60	39	4 pyloric antral ulcers
4*	64	32	3 ,, ,, ,,
5*	12	6	Numerous antral ulcers
6*	16	9	3 antral ulcers, 1 fundic ulcer
7*	46	26	3 pyloric antral ulcers
8*	6	4	2 pin-point antral ulcers
9*	18	10	Antral ulcers
10*	74	43	Numerous antral ulcers
11*	50	29	3 large antral ulcers
12†	92	60	Large antral ulcer, large annular pyloric ulcer
13†	114	72	Large annular pyloric ulcer.
14†	118	74	Perforated antral ulcer, healed annular ulcer
15†	113	83	Perforated pyloric ulcer, antral ulcers

* Creighton regime.
† Modified Stalker regime.

death.

Discussion. In dogs, vagotomy produces a decrease in total volume of acidity[11] and also a marked decrease in motility[11,12] which persist for at least 5½ months.[13] Meek[14] reported a peptic ulcer in 2 out of 13 vagotomized dogs but Beazell and Ivy[15] failed to find an ulcer in 60 vagotomized dogs. It is evident, therefore, that vagotomy alone is not capable of producing peptic ulcer in a high percentage of cases. In view of our results, this probably means that merely the increased exposure of the gastric mucosa to gastric contents is not the prime factor in the formation of cinchophen-ulcer in vagotomized dogs.

Since vagotomy decreases the total volume of acidity, it may be that cinchophen produces an ulcer, not by producing an hyperacidity, but by inactivating one of the protective mechanisms of the stomach. Possibly it affects mucus in some way so as to render it incapable of preventing hydrochloric acid and pepsin from attacking the stomach wall. The importance of mucus as a protective mechanism was pointed out by Whitlow[16] and conclusively demonstrated in man[17] merely by allowing a normal amount of acid and pepsin to attack an area of mucosa deprived of mucus.

Summary and Conclusions. Surgical interruption of the vagus nerves above the diaphragm had no effect whatsoever on the incidence of peptic ulcer produced by cinchophen.

12 Meek, W. J., and Herrin, R. C., *Am. J. Physiol.*, 1934, **109**, 221.

13 Vanzant, F. R., *Am. J. Physiol.*, 1932, **99**, 375.

14 Meek, W. J., personal communication to Beazell, J. M., and Ivy, A. C.[15]

15 Beazell, J. M., and Ivy, A. C., *Arch. Path.*, 1936, **22**, 213.

16 Whitlow, J. E., quoted by Fogelson, S. J., *J. A. M. A.*, 1931, **96**, 673.

17 Wolf, S., and Wolff, H. G., *Human Gastric Function*, Oxford University Press, New York, London, Toronto, 1943, p. 168.

16017 P

Effect of Double Jejunal Lumen Gastrojejunal Anastomosis upon Production of Ulcers by Histamine.

M. E. STEINBERG AND MILTON DE V. BRUNKOW. (Introduced by W. B. Youmans.)

From the Department of Physiology, University of Oregon Medical School, Portland, Oregon.

Von Eiselsberg[1] introduced the exclusion operation for the treatment of duodenal ulcers. The stomach was severed at its distal end. The pyloric segment was closed and the larger proximal segment of the stomach was anastomosed to the jejunum. Devine[2] advocated the division of the stomach at a more proximal level than in the Von Eiselsberg technic. The intent of both of these operations was to exclude the corrosive action of the acid gastric juice from the duodenum and thus bring about the healing of the duodenal ulceration. The results of these operations proved disappointing since jejunal ulcer developed in a majority of the patients operated by the exclusion methods. The Von Eiselsberg and the Devine procedures were tested in the laboratory.[3,4] Jejunal ulcers occurred with regularity when in addition, the alkaline duodenal contents were diverted from the gastrojejunal anastomosis by the Exalto procedure.[5]

The senior author has previously published studies concerning the factor of spasm in the etiology of peptic ulcers.[4,6] In one of these studies a larger jejunal lumen was created by the anastomosis of two parallel jejunal segments in an anti-peristaltic direction.[6] This newly created and enlarged jejunal lumen was anastomosed to the proximal part of the stomach after the closure of the distal segment according to the method of Devine. At the same time the alkaline duodenal contents were diverted from the gastrojejunal anastomosis by the Exalto procedure.[5] None of the 4 dogs operated in this manner and with a double jejunal lumen developed jejunal ulcers. Of the 12 dogs with the Devine and the Exalto operations and with a single lumen gastrojejunal anastomosis, 75% developed jejunal ulcers.[6]

The present studies have been performed for the purpose of determining the effect of this double lumen jejunojejunal anastomosis upon the production of ulcers by histamine-in-beeswax injection. Varco, Code, Walpole and Wangensteen[7] produced peptic ulcers in 100% of dogs with an intact gastrointestinal tract after daily injections of histamine-in-beeswax. Lannin[8] made extensive experiments with the histamine-in-beeswax preparation to determine which of the various gastrojejunal anastomoses gives the most protection against the jejunal ulcer. He was able to produce jejunal ulcers in 100% of dogs subjected to a gastroenterostomy and to the Devine exclusion operation. In our experiments we have performed the gastroenterostomy and the Devine exclusion operation, and in place of the standard single loop gastrojejunal anastomosis we employed the double lumen jejunal anastomosis. Two normal dogs were given daily injections of histamine-in-beeswax, and each of these developed ulcers. Two dogs with double jejunal lumen gastroenterostomy and one with a double jejunal lumen and a Devine exclusion operation were subjected to daily injections of histamine-in-beeswax. One of these 3 animals developed a jejunal ulcer.

1 Von Eiselsberg, A., *Wien. Klin. Wchnschr.*, 1910, 23, 44.

2 Devine, H. B., *Surg., Gynec., and Obst.*, 1925, 40, 1.

3 Winkelbauer, A., *Arch. f. klin. Chir.*, 1926, 143, 649.

4 Steinberg, M. E., *Am. J. Surg.*, 1934, 23, 137.

5 Exalto, J., *Mitt. a. d. Grenzgeb. d. Med. u. Chir.*, 1911, 23, 13.

6 Steinberg, M. E., and Starr, P. H., *Arch. Surg.*, 1934, 29, 895.

7 Varco, R. L., Code, C. D., Walpole, S. H., and Wangensteen, O. H., *Am. J. Physiol.*, 1941, 133, 475.

8 Lannin, B. G., *Surgery*, 1945, 17, 712.

The other 2 with double lumen anastomosis remained in perfect condition and were sacrificed 43 days after the starting of the injections. No evidence of erosions or ulcers were found in the stomach, duodenum or jejunum.

Protocols. Dog No. 1, weight 30 lb. Operation December, 1946. Gastrojejunostomy with a double lumen jejunojejunal anastomosis. April 7, 1947 histamine beeswax, 30 mg daily. April 29, 1947 dog sacrificed. Shallow round jejunal ulcer, 1 cm in diameter with a pin-point penetration.

Dog No. 3, weight 26 lb. Operation, January 16, 1947. Gastroenterostomy with a double lumen jejunojejunal anastomosis. April 7, 1947, daily injections of 30 mg of histamine beeswax. May 20, 1947 dog sacrificed. No ulcers or erosions found.

Dog No. 4, operation, January 22, 1947. Devine exclusion procedure with a double lumen gastrojejunal anastomosis. April 7, 1947, daily injections of 30 mg of histamine beeswax. May 20, 1947, dog sacrificed. No ulcers or erosions found.

Normal dog No. 1, weight 22 lb. May 12, 1947, daily injections of histamine beeswax. May 20, 1947, dog sacrificed. Shallow ulcers about 1 x 1 cm with induration on the lesser curvature of the pylorus.

Normal dog No. 2, weight 18 lb. May 12, 1947, daily injections of histamine beeswax. April 29, 1947, dog sacrificed. Several small ulcers and erosions in the mucosa of the transverse stomach. Two large ulcers of the first part of the duodenum and one shallow ulcer 4 cm distal to the pylorus.

16018

Effect of Urethane on a Transplantable Acute Lymphoid Leukemia.*

L. W. LAW.   (Introduced by C. C. Little.)
(With the technical assistance of Lester E. Bunker, Jr.)

From the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Me.

It has been shown that urethane produces a pronounced reduction in blood leukocytes and in immature myeloid cells in both the human and the mouse in cases of myeloid leukemia.[1,2] In addition definite palliative effects have been reported for human beings.[1] The response in human lymphoid leukemias was less pronounced and more variable than in myeloid leukemias. Recently,[3] the response to urethane of spontaneous lymphoid leu-

kemias in the mouse has been studied and the following encouraging effects were obtained: (1) a pronounced fall in blood leukocytes to or below normal levels and maintenance of these levels with continued therapy (2) a marked reduction in the number of immature cells in the circulating blood. (3) a temporary stabilizing effect on hemoglobin levels. (4) a pronounced diminution in size of subcutaneous lymph nodes, spleen and thymus and (5) a significantly greater life expectancy for urethane-treated cases.

A study of the effect of urethane on several acute and chronic lymphoid and myeloid transplantable leukemias in the mouse has been made. This paper summarizes the response of a transplantable acute lymphoid leukemia, line L825, which arose spontaneously in ♀ 79374 (6 months of age) of the C58 inbred leukemic strain of mice. The leukemic

1 Paterson, E., Haddow, A., Thomas, I. A., and Watkinson, J. M., *Lancet*, 1946, 250, 677.

2 Engstrom, R. M., Kirschbaum, A., and Mixer, H. W., *Science*, 1946, 103, 255.

3 Law, L. W., *Proc. Nat. Acad. Science*, 1947, 33, 204.

TABLE I.
Periodic Peripheral Blood Counts of Urethane-Treated and Untreated C58 Mice Inoculated with Acute Lymphoid Leukemia, Line L825.*

Blood counts	7 days					10 days					12 days				
	Hb—g %	Total leucocytes	Lymphocytes	Neutrophils	Lymphoblasts	Hb—g %	Total leucocytes	Lymphocytes	Neutrophils	Lymphoblasts	Hb—g %	Total leucocytes	Lymphocytes	Neutrophils	Lymphoblasts
Urethane†	15.7	5985	45	51	3.6	13.1	6550	30	66	1.1‡	9.1	7070	37	57	4.0‡
Controls	13.6	29950	48	41	10	9.2	76280	23	37	35	5.3	125750	18	20	58

* Means based upon 9 experimental and 9 control animals except at 12-day period where only 2 control animals were alive for blood studies. Same mice used throughout. Blood obtained from tail vein.
† Dosage: 0.75 ug per g body weight per day.
‡ No lymphoblasts found in blood smears of 4 experimental animals.

animals used were of the 32nd, 33rd and 34th transfer generations of this leukemia and were from 5 to 6 weeks of age.

Leukemia line L825 forms a local tumor following subcutaneous transfer and produces massive and diffuse infiltration into the lymph nodes, spleen, liver and thymus in the terminal stages leading to death of mice in 9 to 10 days. The leukocyte count is elevated after 4 to 5 days and lymphoblasts appear at this time. In terminal stages leukocyte counts of 200,000 (90% lymphoblasts) have been encountered.

Urethane was administered daily in the following dosages to 4 separate groups of mice beginning injections 24 to 48 hours after transfer of leukemic cells: 0.75 mg per g body weight, 0.45 mg, 0.25 mg, and 0.15 mg per g body weight. A 6% aqueous solution was given intraperitoneally. Significant palliative effects noted in the experimental series treated with 0.75 mg per g body weight are reported herein.

The blood leukocyte count of urethane-treated leukemics was maintained at levels below normal throughout the period of inoculations of 0.75 mg urethane per g of body weight, whereas the untreated leukemic animals showed a progressive leukocytosis to a mean leukocyte count of 125,750 at 12 days. The absolute lymphoblast count of urethane treated leukemias was 7%, 0.3% and 0.4% of the absolute lymphoblast count of untreated leukemics at 7, 10 and 12 days respectively following transfer of leukemic cells. At 12 days following treatment the mean absolute number of malignant lymphoblasts in urethane treated mice was 318.8 compared with 72935 in untreated leukemics (Table I).

It has been observed in chronic myeloid leukemias in humans that hemoglobin values rise or remain stationary in the majority of cases. No deleterious effects on the red cell series was observed.[1] In our urethane-treated leukemics the hemoglobin levels did not rise nor were they maintained for any length of time. However there were significantly higher values at each blood count period as compared with untreated controls. When the hemoglobin values fell to 5 g %, death ensued shortly

Exp.	No. animals	Tissue weights in mg/25 g of final body weight*			
		Subcutaneous mass	Spleen	Liver	Subcutaneous lymph nodes‡
Controls	16	1234 ± 93.3†	540.6 ± 41.7	2291.8 ± 158	217.5 ± 13.6
Urethane§	16	230.9 ± 47.6	72.5 ± 6.9	828.8 ± 39.3	62.1 ± 7.3
Difference of means		1003.1 ± 104.7	464.1 ± 42.3	1463 ± 162.7	155.4 ± 15.4
P values		<0.01	<0.01	<0.01	<0.01

* Urethane causes a slight decrease in body weight after many inoculations of sub-anesthetic doses. The means of tissue weights of control and urethane series are weighted equally by using body weights at death of animals.
† Means and standard errors.
‡ Including paired inguinal, axillary, and cervical lymph nodes.
§ Dosage: 0.75 mg per g body weight per day.

thereafter.

Following urethane treatment there resulted a conspicuous decrease in circulating lymphocytes and a corresponding increase in polymorphonuclear leukocytes. This change was evident after 3 daily injections. In view of the fact that this phenomenon occurs in lymphoid as well as in myeloid leukemia the explanation offered for the depression of white bloodcell counts of myeloid leukemias following urethane therapy does not seem tenable.[4]

All urethane-treated mice responded as above described. In 4 mice at the 10 and at the 12 day periods no malignant lymphoblasts could be found in blood smears.

A significantly greater life expectancy was obtained in the urethane-treated (0.75 mg per g daily) series. These experimental animals lived 17.53 ± 0.63 days (25 animals) as compared with 10.37 ± 0.26 days (45 animals) for the controls. The difference of the means of the groups is 7.16 ± 0.69 days where P<0.01. On the other hand leukemic mice given smaller doses of urethane did not live longer than untreated leukemics.

A definite retardation of the growth of the subcutaneous mass was evident at 5 days after transplantation of leukemic cells in the urethane series. All urethane-treated mice were negative at this time whereas control leukemic mice showed definite nodules. At 7 days the mean tumor size of urethane-treated animals was 0.98 cm (sum of 2 axes)

and of control animals 3.2 cm. There was a profound difference in tumor weights obtained at death of the animals. (Table II). Infiltration into the liver, spleen and subcutaneous lymph nodes as determined by organ weights and by histological study was slight in the urethane-treated series. Urethane-treated mice showed a mean loss in weight of nearly 2 g after 14-15 days of daily injections. It is evident that the loss of weight *per se* in the experimental animals producing some splenic and lymphoid atrophy did not result in the significantly lower organ weights for the following reasons: (1) At 10-12 days following initiation of urethane-therapy, at which time there was no loss in body weight, massive and diffuse infiltration into spleen and subcutaneous lymph nodes was not conspicuous. (2) Organ weights in urethane-treated leukemics were not significantly different from similar organ weights of normal C58 mice of the same age and body weight. (3) It is evident from histological studies that cell destruction of infiltrating lymphoblasts is occurring. Complete histological studies will be given later.

Summary. Urethane, administered intraperitoneally in subanesthetic dose, 0.75 mg per g of body weight per day in leukemic mice transplanted with leukemic cells of the 32nd to 34th transfer generations of an acute lymphoid leukemia, produces the following profound palliative effects: (1) The blood leukocytes are maintained at leukopenic levels. (2) The immature lymphoblasts are main-

4 Kirchbaum, A., and Lu, C. S., PROC. SOC. EXP. BIOL. AND MED., 1947, 65, 62.

tained at significantly lower levels than control leukemics and in some cases disappear entirely from the peripheral blood. (3) Hemoglobin values remain at higher levels in the urethane-treated animals throughout treatment. (4) Local subcutaneous growth of the tumor mass is retarded. Some infiltration of lymphoblasts into spleen, thymus and lymph nodes occurs, however this is not of the characteristic massive and diffuse type. Lymphoblast destruction is evident. (5) A statistically significant greater life expectancy in urethane-treated leukemics is obtained.

16019

Comparison of Rates of Penetration of Unwashed and Washed Spermatozoa in Cervical Mucus.*

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The evidence at hand indicates that the secretion of cervical mucus is under hormonal control.[1-4] It is most abundant when ovulation is believed to occur, *i.e.* at midinterval in the usual 28-day cycle.[5-7] At this time the mucus possesses its lowest viscosity and cellularity,[8] highest water content,[9] and is well supplied with glycogen and other potential reducing substances.[9] *In vitro* experiments show that it is during midcycle that the cervical mucus is most readily penetrable by spermatozoa,[6,8] probably due to the physical and chemical properties that prevail at this time. MacLeod

has made the observation that human spermatozoa require glucose or a like monosaccharide for their metabolism and prolonged activity.[10] The fresh semen itself contains some 300 mg per 100 cc of reducing substance expressed as glucose.[11,12] Since spermatozoa probably carry with them little of this extracellular nutriment, they may find in their new environment the necessary substrate for their subsequent metabolism. Enzymes may play an important rôle in the utilization by the spermatozoa of the substances in cervical mucus, but this conjecture constitutes a separate study.

This report is concerned with an endeavor to ascertain the effect of washing of the spermatozoa on their ability to penetrate a column of cervical mucus *in vitro*.

Material and Methods. Subjects: Healthy young women with normal menstrual cycles and pelvic findings supplied the mucus which was collected from the cervical canal by aspiration.[5] Semen specimens were obtained from healthy young donors by manual stimulation. In the evaluation of these specimens consideration was given to the count and

1 Guttmacher, A. F., and Shettles, L. B., *Human Fertil.*, 1940, 5, 4.

2 Bennett, H. G., Jr., *Am. J. Obst. and Gynec.*, 1942, 44, 296.

3 Abarbanel, A. R., *Trans. Am. Soc. for Study of Sterility*, 1946.

4 Pommerenke, W. T., and Viergiver, E., *J. Clin. Endocrin.*, 1946, 6, 99.

5 Séguy, J., and Simonnet, H., *Gynéc. et obst.*, 1933, 28, 657.

6 Lamar, J. K., Shettles, L. B., and Delfs, E., *Am. J. Phys.*, 1940, 129, 234.

7 Viergiver, E., and Pommerenke, W. T., *Am. J. Obst. and Gynec.*, 1944, 48, 321.

8 Viergiver, E., and Pommerenke, W. T., *Am. J. Obst. and Gynec.*, 1946, 51, 192.

9 Viergiver, E., and Pommerenke, W. T., *Am. J. Obst. and Gynec.*, in press.

10 MacLeod, J., *Endocrinology.* 1941, 29, 583

11 Hotchkiss, R. S., Brunner, E. K., and Grenly, P., *Am. J. M. Sci.*, 1938, 196, 362.

12 Huggins, C. B., and Johnson, A. A., *Am. J. Physiol.*, 1933, 103, 574.

TABLE I.
Rate of Penetration of Unwashed and Washed Spermatozoa in Cervical Mucus.

	No. of specimens	Range of rate of penetration, mm/min	Avg rate of penetration, mm/min
Preovulatory phase			
Unwashed	8	0.0-0.7	0.3
Washed	5	0.0-0.4	0.2
Ovulatory phase			
Ascending			
Unwashed	13	0.6-2.3	1.3
Washed	10	0.5-1.9	1.0
Peak			
Unwashed	6	1.3-2.0	1.7
Washed	10	1.0-2.7	2.0
Descending			
Unwashed	5	0.2-1.8	0.8
Washed	3	0.7-1.4	1.0
Postovulatory phase			
Unwashed	7	0.0-1.5	0.5
Washed	6	0.0-0.6	0.3

morphology.

Preparation of Suspensions of Washed Spermatozoa. One volume of semen and 5 volumes of Ringer's solution were mixed and then centrifuged at approximately 1500 r.p.m. for 10 minutes. The packed spermatozoa were then resuspended in fresh Ringer's solution, the final mixture being brought up to the original volume of semen used. Observations on the counts and morphology were also made on these suspensions. The washing and centrifugation of the spermatozoa resulted in some diminution in the number of motile forms and also in the destruction of some of the cells.

Penetrability. The rate of pentrability was determined by the method of Lamar, Shettles and Delfs.[6] A column of mucus followed by a column of fresh semen or of washed spermatozoa suspended in Ringer's solution was drawn into a capillary tube, leaving a small bubble between the 2 media to serve as a marker. These tubes were then placed on glass slides and covered with mineral oil to reduce refraction for observation under the microscope. By means of a stop watch and a calibrated mechanical stage the rate at which the spermatozoa invaded the cervical mucus was determined.

Results. The mucous cycles were arbitrarily divided into 3 phases: preovulatory, ovulatory, and postovulatory, the ovulatory phase consisting of those days in midcycle during which the amount of mucus was greatly increased and the cellularity decreased. In the normal cycle the amount of mucus increases for 2 to 3 days in the beginning of the ovulatory phase, reaches a peak which is maintained from 1 to 2 days, and then decreases for 2 to 3 days before reaching the postovulatory level.[7,8] Since it has been shown that the rate of penetrability of spermatozoa also follows this general pattern,[6,8] the ovulatory phase was further subdivided into the ascending side, the peak, and the descending side of the curve.

Reference to Table I shows that the rates of penetration of the unwashed spermatoza,[6,8] are in accord with previous observations.[6,8] It can likewise be seen that washing and centrifuging of the spermatozoa did not appear to modify appreciably their ability to invade the mucus. In some tests the spermatozoa, both unwashed and washed, were entirely stopped in their attempt to penetrate the cervical mucus obtained during the pre- and postovulatory phases. However, the average rate of penetration varied from 0.2 to 0.5 mm/min. The highest rate of penetration, *i.e.* from 1.7 to 2.0 mm/min., occurred in those specimens of mucus collected during the ovulatory phase, with some diminution in rate as the collections of mucus departed on either side from the peak.

In these studies measurements of the rate

of penetration were begun promptly after the spermatozoa were brought into contact with the column of mucus. In many cases the spermatozoa on finding the mucus medium particularly favorable, *i.e.* during the ovulatory phase, could be observed to pass through the entire length of the mucus column, an average distance of 25 mm, without apparent deceleration. However, in the pre- and post-ovulatory phases of the cycle when the mucus was more viscid and cellular, the entire distance to which they could penetrate averaged only 1.4 mm. There was no apparent difference between the distances penetrated by the unwashed and the washed spermatozoa.

In all, 42 semen specimens coming from 4 donors were used. These specimens ranged in volume from 0.6 to 4.2 cc and in count from 34 million to 262 million per cc. We were unable, in these studies, to correlate the degree of penetrability of the mucus with the concentration of spermatozoa, either unwashed or washed.

Summary. This study indicates that washing human spermatozoa with Ringer's solution and resuspending the washed spermatozoa in this solution does not impair their ability to penetrate a column of cervical mucus *in vitro.*

16020

Uneven Distribution of Glycogen in the Liver.[*]

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A strikingly uneven distribution of glycogen in the liver of rabbits was found in the course of experiments in which an attempt was made to follow with biopsies the variation in liver glycogen under various conditions. Irregularities in the intracellular ("glycogen flight") and intralobular[1] distribution of glycogen have been known for some time. Recently, Deane, Nesbett and Hastings[2] have found significant differences in the glycogen content of the two lobes. Since it is obvious that such observations tend to limit the value of liver biopsies in judging the metabolic state of the organ, a short report appears to be warranted.

Experimental. Rabbits were fasted for 18 to 24 hr and laparotomized under sodium pentothal anesthesia. On gross inspection no difference in the appearance of the lobes of the liver was noted. Simultaneous wedge shaped specimens weighing 1000 to 1500 mg were removed from different parts but usually from the same lobe of the liver. Where biopsies were taken at intervals Gelfoam proved very helpful in the control of bleeding. Subsequent biopsies were taken from sites far away from that of the previous resection.

The present report is concerned only with variations observed in simultaneous biopsies. The specimens were cut into 3 portions. The middle slice was fixed promptly in chilled 90% alcohol or Bouin's fluid, embedded, and the sections stained with Best's carmine, Bauer-Feulgen's method[3] and a silver stain.[4] The two lateral portions were weighed and dropped immediately in 30% KOH for the chemical determination of glycogen according to Good, Kramer and Somogyi;[5] the glucose

1 Edlund, Y., and Holmgren, H., *Z. f. mikr-anat. Forsch.,* 1940, 47, 467.

2 Deane, H. W., Nesbett, F. B., and Hastings, A. Baird, Proc. Soc. Exp. Biol. and Med., 1946, 63, 401.

3 Bauer, H., *Z. f. mikr-anat. Forsch.,* 1933, 33, 143.

4 Gomori, G., *Am. J. Clin. Path.,* 1946, 10, 177.
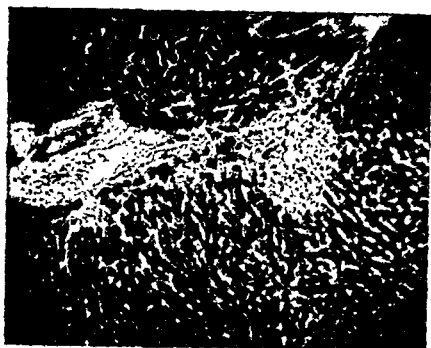
FIG. 1.
Rabbit 52E, biopsy 1. $\times$ 80.

FIG. 3.
Rabbit 48, biopsy 3. $\times$ 25.
All slides stained with Bauer-Feulgen's stain.
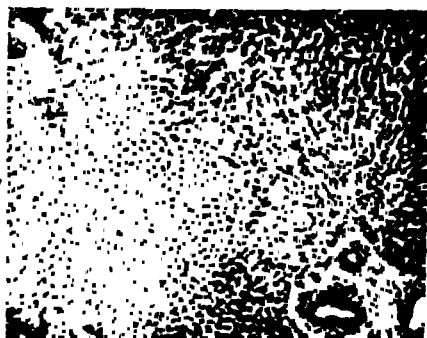
FIG. 2.
Rabbit 52E, biopsy 3. $\times$ 80.

chemical findings, as shown in Fig. 1 and 2. Fig. 3 demonstrates furthermore that even in the very same slide extensive areas of high and low glycogen content may be found side by side.

It should be mentioned that the distribution of fat in the sections, unlike that of glycogen, appeared to be quite uniform.

formed was determined by the colorimetric method of Folin and Malmros,[6] a photo-electric colorimeter being used.

Table I shows the marked variation in the amount of glycogen found in simultaneous biopsies, the range sometimes exceeding several 100%. The histologic pictures, as far as one can judge from the extent and intensity of the stain, were in good agreement with the

TABLE I.
Variation in Glycogen Content of Simultaneous Liver Biopsies. (% of wet weight.)

Rabbit No.	Biopsies		
	1	2	3
48	0.23	0.4	1.6
51 E	0.74	0.9	0.65
52 A	1.6	2.4	—
52 E	1.8	1.5	0.5
53 A	4.2	5.1	—
53 E	2.6	3.5	1.8

Summary. Great caution is warranted in judging the metabolic state of the liver on the basis of the glycogen content of biopsies since variations exceeding several 100% may be observed in simultaneous biopsies taken from the same lobe of the rabbit's liver.

[5] Folin, O., and Malmros, H., J. Biol. Chem., 1929, 83, 115.

[6] Good, A., Kramer, H., and Somogyi, M., J. Biol. Chem., 1933, 100, 485.

16021 P

Temperature Level and the Growth of Embryo and Tumor of Tumor-Bearing Eggs.

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The technique of growing tumor tissue in embryonated eggs has been routine in this laboratory for more than 4 years. The use of the yolk sac method of inoculation has made it comparatively simple to maintain rat and mouse tumors indefinitely by egg to egg inoculations.[1,2]

The size of the tumors grown in this manner were of the same order as those obtained by transplant in the host animal. This continued to be so after as much as 114 transplant-generations or about 4 years continuously in eggs. Apparently the egg environment satisfies all the requirements of malignant tissue for vigorous growth.

The inoculation of eggs through the yolk sac results in tumor implantation on the yolk sac inner wall.[3] In this situation the growth of the tumor presents a minimum of interference with the development of the chick embryo. Tumor tissue and chick tissue grow together sharing a common blood supply. It is not uncommon for the egg-grown tumor to weigh as much as 6 g at the 17th day of egg incubation, while the chick embyro normally weighing about 20 g at this stage is reduced to 10 or 12 g.

The egg environment is relatively stable but a certain amount of manipulation will be tolerated by the chick embryo. This is especially so with respect to temperature. Incubating eggs will withstand considerable

variation in temperature and still survive to the 17-18 day period.

The present paper is concerned with a study of the comparative reactions of tumor and chick tissues grown together to temperatures above and below that required for normal incubation.

Experimental. Embryonated eggs on the 4th day of incubation were inoculated with dba mouse mammary carcinoma. The eggs of one series of inoculations were divided into 3 groups and incubated from the time of implantation until the termination of the experiment at 3 different temperatures. One group representing the control was kept at 99-100° F, a second group was incubated at 96-97°F, and the third group was maintained at a temperature of 103-104°F. At the 18th day of incubation, or 14 days after tumor inoculation, the embryos and tumors of the surviving eggs were harvested and weighed. A total of 1179 eggs was inoculated with tumor tissue for this research involving 17 separate experiments.

The results are given in Table I. Statistical validity of the figures given has been checked and found to be beyond question.

The chick embryo was less affected by the higher- and lower-than-normal incubating temperatures than was the egg-cultivated tumor. This was particularly striking for the temperature range. 103-104°F, at which the chick embryos averaged a reduction in weight of 12% while the tumors grown in these eggs were reduced in size 50% as compared with their respective weights at the control temperature.

Previous work has demonstrated that in eggs inoculated by the yolk sac method the size of the embryo is generally conditioned by the size of the tumor.[4] A large vigorous

[1] Taylor, A., Hungate, R. E., and Taylor, D. R., *Cancer Research*, 1943, 3, 537.

[2] Taylor, A., Thacker, J., and Pennington, D., *Science*, 1942, 96, 342.

[3] Hungate, R. E., Taylor, A., and Thompson, R. C., *Cancer Research*, 1944, 4, 289.

[4] Kynette, A., Taylor, A., and Thompson, R. C., *Univ. of Texas Publication No. 4507, Cancer Studies*, 1945, pp. 65-75.

TABLE I.

Effect of Temperatures Above and Below Normal Incubation on Embryo and Tumor Weights of Tumor-Bearing Eggs.

No. exp.	Incubation temperature (°F)	No. eggs inoculated	No. eggs surviving	Avg. chick size (g)	Exp. chicks, control = 100	Avg. tumor size (g)	Exp. tumor control = 100
17 (control)	99-100	397	165	11.8 ± 1.8	100	1.0 ± .4	100
17	96-97	392	145	10.1 ± 1.2	85.6	.6 ± .2	60
17	103-104	390	55	10.4 ± 1.0	88.1	.5 ± .3	50

tumor frequently occurs in the egg together with an anemic undersized chick embryo. It appears that the relation of the chick embryo to the tumor implanted in its yolk sac is comparable to the relation of the host mouse to a tumor growing from a transplant.

It seems probable therefore that the reduc-tion in size of the egg-grown tumors in response to temperature level above and below 99-100°F was a direct effect. Further, the data indicate that the egg-cultivated tumors are more sensitive to this factor than the sup-porting embryos.

16022

The Tuberculin Reaction. I. Passive Transfer of Tuberculin Sensitivity with Cells of Tuberculous Guinea Pigs.*†

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(Introduced by Charles A. Evans.)

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It has been established that the "tuberculin type" of sensitivity is distinct from the ana-phylactic or Arthus type of sensitivity and from the atopic type of sensitivity dependent upon Prausnitz-Küstner antibody.[1-6] Although it is probable that the tuberculin type of sensitivity depends upon an antibody, the existence of such an antibody has not been established with certainty.

That tuberculin type sensitivity is depend-ent upon antibody is indicated by the similar-ities it shows to other sensitivities known to be dependent upon antibody, namely, sim-ilarities relating to specificity, incubation time, desensitization, anamnestic reaction, and the correlation between the ability of various animal species to form ordinary anti-body and to develop tuberculin sensitivity. Supporting evidence is also provided by the observation that blockage of the reticulen-dothelial system which depresses the forma-tion of ordinary antibody to an injected protein, likewise suppresses the development of tuberculin sensitivity.[7] In addition, it has been found that the use of adjuvants such as

1 Holst, P. M., Tubercle, 1922, 3, 337.

2 Rich, A. R., Lewis, M. R., Bull. Johns Hopkins Hosp., 1932, 50, 115.

3 Rich, A. R., The Pathogenesis of Tuberculosis, p. 412, Charles Thomas, Springfield, Ill., 1944.

4 Aronson, J. D., J. Immunol., 1933, 25, 1.

5 Moen, J. K., and Swift, H. F., J. Exp. Med., 1936, 64, 339.

6 Heilman, D. H., Feldman, W. H., and Mann, F. C., Am. Rev. Tub., 1944, 50, 344.

petroleum jelly (Petrolatum) and paraffin oil, enhances the ability of heat-killed *Mycobacterium tuberculosis* to engender the development of tuberculin sensitivity[8,9] and, in like manner, of protein antigen to engender ordinary antibody:[10]

Many attempts have been made to accomplish passive transfer of tuberculin sensitivity.[7,11-22] It is notable that in the few reports where success has been claimed the results have been for the most part irregular and difficult to reproduce. In many instances adequate controls were not included or recognition apparently given to the difference between the true tuberculin reaction and the Arthus and anaphylactic reactions.

Helmholz[11] reported successful homologous passive transfer of tuberculin sensitivity with the defibrinated blood of tuberculous guinea pigs.

Bail[12] also reported homologous passive transfer of tuberculin sensitivity by the intraperitoneal injection of ground uncaseated spleen, liver and lymph glands of tuberculous guinea pigs. When the recipient animals were given Old Tuberculin 24 hours later by the intraperitoneal, subcutaneous, and intrapleural routes, most of the animals died within 28 to 31 hours. Bail attributed this to the systemic tuberculin reaction. Control animals given the ground spleen and liver of nontuberculous donors and injected with O.T. in the same manner survived. Cutaneous sensitivity was absent in the recipient animals. However, this is not surprising since the animals were in a very weakened condition and may have been nonspecifically non-reactive.

Joseph,[13] who attempted to repeat Bail's experiments concluded that the results observed by the latter did not prove passive transfer of tuberculin sensitivity. This was based on the observation that the tissue preparations of the donor tuberculous animals were in themselves very toxic. He assumed that the additional toxicity of the tuberculin injected may have been sufficient to kill the recipient animals without the true tuberculin reaction being involved.

Onaka[14,15] confirmed the observations of Bail and of Helmholz but did not agree with the conclusions drawn by the latter.

Massol, Breton and Bruyant[17] reported homologous passive transfer of tuberculin sensitivity with the citrated blood of tuberculous guinea pigs. They employed the technique of reversed passive transfer and used increases in body temperature as an index of tuberculin sensitivity.

Zinsser and Mueller[18] reported irregular success with heterologous passive transfer of tuberculin sensitivity to guinea pigs with the serum of tuberculous rabbits. In successful cases cutaneous reactions could be elicited 3 days following the transfer. However, positive results were infrequent and could not be reproduced at will.

Freund[7] failed to accomplish homologous local passive transfer of tuberculin sensitivity by use of blood serum and organ extracts of tuberculous guinea pigs.

Chase[22] has recently accomplished homologous passive transfer of tuberculin sensitivity in guinea pigs with living cells. The fundamental importance of this finding cannot be overemphasized. It provides the strongest

7 Freund, J., *J. Immunol.*, 1926, **11**, 383.

8 Saenz, A., *C. R. Soc. de biol.*, 1935, **120**, 870.

9 Freund, J., Casals-Ariet, J., and Genghoff, D. S., *J. Immunol.*, 1940, **38**, 67.

10 Freund, J., and McDermott, K., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 548.

11 Helmholtz, H. F., *Ztsch. Immunitatsforschg.*, 1909, **3**, 371.

12 Bail, O., *ibid.*, 1909, Orig. IV, **1**, 470.

13 Joseph, K., *Beit. z. Klinik der Tuberk. von Brauer*, 1910, **17**, 461.

14 Onaka, M., *Z. Immunitatsforschg.*, 1910, **5**, 264.

15 Onaka, M., *ibid.*, 1910, **7**, 507.

16 Kraus, R., Loewenstein, E., and Volk, R., *Cent. fur Bakt.*, 1911, **1**, 361.

17 Massol, L., Breton, M., and Bruyant, L., *C. R. Soc. de biol.*, 1913, **74**, 185.

18 Zinsser, H., and Mueller, J. H., *J. Exp. Med.*, 1925, **41**, 159.

19 Hanks, J. H., *J. Immunol.*, 1935, **28**, 105.

20 Dienes, L., and Schoenheit, E. W., PROC. SOC. EXP. BIOL. AND MED., 1926, **24**, 32.

21 Dienes, L., *J. Immunol.*, 1927, **14**, 43.

22 Chase, M. W., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 134.

evidence, thus far presented, that tuberculin sensitivity depends upon antibody and is not the result of some unknown mechanism capable of simulating antigen-antibody reactions.

Holst[1] observed that tuberculin exerts a toxic action on leucocytes derived from tuberculous animals as evidenced by decreased phagocytic power and loss of differentiation between nucleus and cytoplasm. He clearly established the difference between tuberculin sensitivity and sensitivity due to ordinary antibody by demonstrating that horse serum does not exert an *in vitro* toxic action on the leucocytes of guinea pigs sensitized to that antigen.

Rich and Lewis[2] observed that tuberculin is toxic to the cells of tissue cultures prepared from tuberculin-sensitive animals. Their work was confirmed by Aronson,[23] who noted that the toxic effect of tuberculin on tissue cultures is highly specific.

Aronson[4] made the additional observation that in contrast to this susceptibility of tuberculin-sensitive tissues, tissue cultures from Arthus-sensitive animals are unaffected by specific antigen. This was later confirmed by Rich.[24]

Knowledge of the cytotoxic effect of tuberculin on cultured cells derived from tuberculin-sensitive animals has been greatly extended by the studies of Moen and Swift[5] and Heilman, Feldman and Mann.[6]

The present report is the outcome of some of our initial studies on the nature of the tuberculin reaction. The plan of our investigations necessitated the passive transfer of tuberculin sensitivity. Hence, preliminary trials were made to accomplish passive transfer by the method reported by Chase.[22] In the earliest of these trials, it became apparent that success may be irregular, due in all probability to as yet unrecognized factors.

Experiments. The methods employed were essentially the same as those used by Chase,[22] except that in some of the experiments the donor guinea pigs were subjected to a superimposed infection with the B.C.G. strain of *M. tuberculosis.* The animals were secured from local sources and were of unknown pedigree. They averaged approximately 600 to 800 g in weight. In the first experiments, sensitization of the donors was accomplished by the subcutaneous injection of 25 mg (wet weight) of heat-killed *M. tuberculosis* H37 Rv in paraffin oil. In some of the subsequent experiments, the animals were sensitized by the injection of heat-killed organisms followed several months later by the intraperitoneal injection of 0.5 mg of living B.C.G. and in others by a single intraperitoneal injection of 5 to 10 mg of living B.C.G.

Five to 9 weeks following the sensitizing injections, the animals were skin-tested and *the most highly sensitive animals* injected intraperitoneally with 30 ml of sterile paraffin oil. Forty-eight hours later, the animals were killed and the cells of the resulting exudate were collected, washed in guinea pig serum-Tyrode solution and injected intraperitoneally into normal mature light-skinned recipient animals. Approximately 90% of the cells were alive as indicated by supravital staining with neutral red and consisted of approximately 10% polymorphonuclear cells, 47% lymphocytes, and 42% large mononuclear cells. Washed cells of the buffy coat of the minced spleens were injected intraperitoneally into other recipients. Each recipient received either the pooled peritoneal or splenic cells of from 2 to 8 donors. The cell volumes transferred to each recipient ranged from 0.4 to 1.0 ml. Control recipient animals received similar preparations from donor controls which had been given a "sensitizing" injection of paraffin oil without tubercle bacilli.

Forty-eight to 72 hours after cell transfer, all animals, including a set of normal controls, were skin-tested with O.T. in dilutions ranging from 1:10 to 1:100 or with deglycerinated O.T. employing the lowest dilution which failed to give a reaction in normal animals.

A total of 48 donors and 18 recipients were used in the various experiments. The results of the first experiment, in which donors sensitized with heat-killed tubercle bacilli were

23 Aronson, J., *J. Exp. Med.*, 1931, **54**, 387.

24 Rich, A. R., *The Pathogenesis of Tuberculosis,* p. 409, Charles Thomas, Springfield, Ill., 1944.

used, were negative except for a slight reaction in one recipient. The reaction in this animal became apparent after 12 hours and reached its height by 24 hours at which time it presented a 15 mm area of 2+ erythema and edema.

A careful consideration of this trial led us to believe that the most probable cause for our lack of success was that the strain of donor animals we used possessed a low ability for developing sensitivity. They showed 4+ reactions to 1:100 O.T. but only 3+ reactions to 1:100 O.T.

In an effort to render the donors more sensitive we next sensitized the animals with either heat-killed *M. tuberculosis* H37Rv and living B.C.G. or living B.C.G. alone, as outlined above. The majority of these animals developed a strong sensitivity. They reacted to 1:1000 O.T. with extensive necrosis and gave positive reactions to deglycerinated O.T. with dilutions of 1:40,000 and higher. The yield of peritoneal cells from these donors was about double that of the donors sensitized with heat-killed tubercle bacilli. Most of the recipients that received cells from these animals gave positive reactions to tuberculin. The reactions in animals sensitized with peritoneal cells were strong and of a typical tuberculin type. They were commonly negative for the first 10 hours, became fully developed by about the 30th hour and consisted of 15 mm areas of 3+ erythema, edema and induration, with a sharp central 5 mm zone of ischemic blanching. The reactions usually began to fade by the 40th to 50th hour, but were still apparent at 72 hours or longer. In several instances the early reactions were so intense that necrosis was anticipated but never developed. Intense reactions were elicited with dilution of O.T. as high as 1:100.

The animals that received splenic cells were usually less sensitive. They gave typical but less intense tuberculin reactions which lacked central blanching and faded earlier. The skin tests of most of the control animals were negative. The few controls which reacted presented small areas of 1+ erythema and edema which clearly differed from the reactions of the test animals by fading earlier and being devoid of central blanching. Fading was evident by the 24th hour and complete by the 48th hour.

The transferred cutaneous sensitivity was of short duration. Skin tests made 5 days after cell transfer were negative.

A preliminary attempt was made to elicit the systemic tuberculin reaction in two of the recipient animals by the intraperitoneal injection of 2 ml and 0.6 ml of O.T. respectively. The material was prepared for injection by dilution with several volumes of physiological saline. The animals reacted with a delayed type of shock which came on after about 3 hours. They were in severe shock by the 8th hour. The animal that received 2 ml of O.T. was dead at 24 hours and the other animal recovered. A second similar dose of O.T. administered to this animal 24 hours after the first was without effect. Normal control animals that were similarly injected remained unaffected.

Discussion. As Chase has emphasized, the successful passive transfer of tuberculin sensitivity in the guinea pig apparently depends upon the transfer of a large number of living leucocytes from highly sensitive donor animals.

A question of much interest and importance is whether an antibody is involved and what its nature may be. If an antibody is responsible for reactions to tuberculin, it is important to explain the apparent sessile existence of this "tuberculin antibody." Perhaps one of its properties is that it is avidly absorbed by tissues. This could account for its wide distribution and apparent sessile existence since the quantity of antibody in the circulation may thus be kept at such low levels as to be undetectable. A less-likely alternative is that all cells exhibiting sensitivity are capable of forming the antibody which remains fixed in the cells.

There appears to be little doubt that the transfer accomplished in the present experiments was passive and not active because of the short incubation period and transient nature of the sensitivity. In fact, the duration of tuberculin sensitivity for only a few days is so much shorter than the Arthus, anaphy-

lactic and Prausnitz-Kustner sensitivities as to indicate that a different and perhaps quite labile antibody must be concerned. The fact that passive transfer of tuberculin sensitivity has been accomplished only with living cells and only after an incubation period of two to three days suggests that the sensitivity may be principally due to antibody elaborated by such cells during their residence in the re-cipient rather than to preformed antibody.

Summary. Homologous passive transfer of tuberculin sensitivity was accomplished with the peritoneal and splenic cells of guinea pigs infected with the B.C.G. strain of *Mycobacterium tuberculosis.*

The possible mechanism and factors involved in passive transfer of tuberculin sensitivity are discussed.

16023

Effects of X-ray Irradiation on Viscosity of Synovial Fluid.

CHARLES RAGAN, CHARLOTTE P. DONLAN, JAMES A. COSS, JR. AND AUDREY F. GRUBIN.
. (Introduced by R. F. Loeb.)

From the Departments of Medicine and Radiology of the College of Physicians and Surgeons, Columbia University, and the Edward Daniels Faulkner Arthritis Clinic of the Presbyterian Hospital, New York.

The treatment of arthritis by X-rays advocated in the European[1] and English[2] literature since 1930 is difficult to evaluate. However, in 1941, Smyth, Freyberg and Peck[3] demonstrated conclusively that a favorable response to X-ray therapy is obtained in ankylosing spondylitis. Many confirmatory reports have followed this observation.[4] The mechanism whereby improvement follows roentgenotherapy in such cases is not apparent.

Recently, two papers[5,6] have appeared describing a reduction in the viscosity of thymonucleic acid after irradiation, depending in degree upon the total X-ray dosage given. This is in agreement with the principles outlined by Colwell[7] in his review of work dating from 1912, to the effect that a diminution in viscosity of organic colloids such as egg-white, serum and starch followed exposure to X-ray. The reason such a change occurs is still not clear. In the case of thymonucleic acid,[6] evidence was presented that the change induced by X-radiation did not involve a splitting of primary linkages or a rearrangement of the configuration which made it susceptible to enzymatic attack.

The viscosity of normal human synovial fluid is very high.[8,9] The viscosity of joint fluids obtained from the knees of patients with rheumatoid arthritis tends to be greater in the chronic than in the acute stage, and to approach the viscosity of normal synovial fluid.[10]

The present study records observations which show that irradiation of joint fluids

1 Kahlmeter, G., *Brit. J. Actinotherapy,* 1930, 5, 93.

2 Scott, S. G., *Proc. Roy. Soc. Med.,* 1932, 25, 972.

3 Smyth, C. J., Freyberg, R. H., and Peck, W. S., *J. A. M. A.,* 1941, 116, 1995.

4 Kuhns, J. G., and Morrison, S. L., *N. E. J. Med.,* 1946, 235, 399.

5 Sparrow, A. H., and Rosenfeld, F. M., *Science,* 1946, 104, 245.

6 Taylor, B., Greenstein, J. P., and Hollaender, A., *Science,* 1947, 105, 263.

7 Colwell, H. A., *The Method of Action of Radium and X-rays on Living Tissue,* Oxford Univ. Press, 1935.

8 Schneider, J., *Biochem. Z.,* 1925, 160, 325.

9 Ragan, C., PROC. SOC. EXP. BIOL. AND MED., 1946, 63, 572.

10 Unpublished observation.
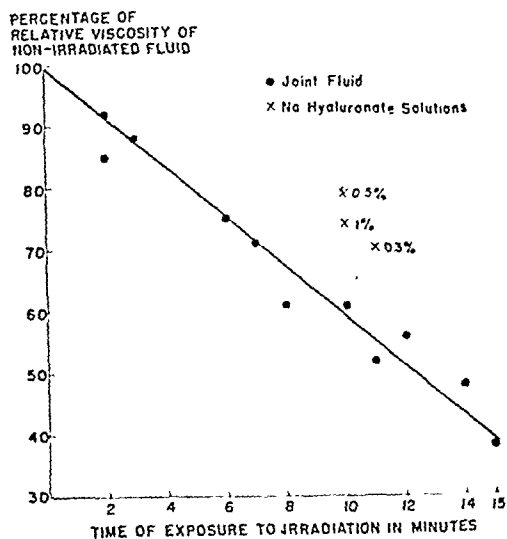
FIG. 1.
Effect of X-ray irradiation on viscosity of joint fluid and Na hyaluronate solutions.

diminishes their viscosity and that the decrease bears a direct relationship to the amount of irradiation.

Methods. Relative viscosity was measured in 5 cc Ostwald viscosimeters in a water bath at $21 \pm 1°C$. Total protein concentrations of the synovial fluid were determined with a gradient tube method.[9,11] The amount of hyaluronic acid present was measured by a turbidimetric method[12] similar to that described by Seastone.[13] Irradiation was furnished by a Phillips contact machine delivering 9290 r/minute. Material to be irradiated was placed in a 50 cc glass centrifuge tube in contact with the treatment tube of the X-ray machine. The average roentgen delivery to the fluid was 334 r/minute.* Because of the type of irradiation, this estimate is only approximate.

The results of the irradiation of 4 patho-

logical knee joint fluids[†] are shown in Fig. 1. Their relative viscosities were 214, 59.4, 42.7 and $22.5 \times H_2O$. When these were plotted against time of exposure, the correlation between the time of irradiation and the percentage decrease in viscosity was good.

The effect of irradiation on solutions of pure sodium hyaluronate[‡] of varying concentrations was less than the effect on joint fluid (Fig. I.). Upon irradiation of the pure solutions, no increase in titratable acidity above that of the unirradiated controls was demonstrable, which would imply that there was no oxidative degradation. In irradiated joint fluids, no appreciable decrease in the amount of hyaluronate was detectable turbidimetrically nor was there any change in the total protein content of the fluid (Table I.). A small difference in the enzymatic hydrolysis of the irradiated and control samples was detectable viscosimetrically. The half-time for the irradiated sample was 14.5 minutes, and of the control 17 minutes, when equal amounts (0.05 mg equal to 7 viscosity reducing units) of bull testis hyaluronidase were used. From this one may assume that no profound change in the hyaluronic acid molecule had occurred.

An effort was made to detect the continuing fall in viscosity on standing after irradiation, as described by Taylor *et al.*[6] for thymonucleic acid. Some indication of this was seen in the preparations of pure hyaluronate, but not in the joint fluid preparations.

Discussion. Irradiation causes a decrease in viscosity of joint fluid. It was impossible to determine the exact amount of irradiation delivered under our conditions, but the decrease bore a direct relation to the time of exposure. The manner in which the molecule is changed is not clear from these observations. That purified hyaluronic acid is

11 Lowry, O. H., and Hunter, T. H., *J. Biol. Chem.*, 1945, 159, 465.

12 Meyer, K., *Physiol. Rev.*, in press.

13 Kass, E. H., and Seastone, C. V., *J. Exp. Med.*, 1944, 79, 319.

* This was calculated with an ionization chamber in the fluid at 3 sites: 1. Nearest the X-ray tube. 2. In the center of the centrifuge tube. 3. At the side the greatest distance from the X-ray tube. The results of the three readings were averaged.

† Two of the fluids were obtained at different times from a patient with rheumatoid arthritis. One was obtained from a patient with an undiagnosed arthritis of the knees, possibly associated with lymphogranuloma inguinale. One was a pooled sample containing knee joint fluid from several patients with rheumatoid arthritis.

‡ Obtained from Dr. K. Meyer.

lactic and Prausnitz-Kustner sensitivities as to indicate that a different and perhaps quite labile antibody must be concerned. The fact that passive transfer of tuberculin sensitivity has been accomplished only with living cells and only after an incubation period of two to three days suggests that the sensitivity may be principally due to antibody elaborated by such cells during their residence in the re-cipient rather than to preformed antibody.

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8 Schneider, J., *Biochem. Z.*, 1925, 100, 325.

9 Ragan, C., PROC. SOC. EXP. BIOL. AND MED., 1946, 63, 572.

10 Unpublished observation.

16024

Lack of Effect of Secondary Liver Extract (No. 55) on Absorption of Radioactive Iron.*

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Introduction. It has long been recognized that in anemias due to chronic hemorrage, either experimental or pathological, iron is efficient in the restoration of the normal blood picture. An inability on the part of the body to form enough of the porphyrin fraction of the hemoglobin molecule has never been established, even under the most stringent conditions. It is with extreme difficulty[1] that one can demonstrate the limitation of hemoglobin production under experimental conditions through the lack of protein for the formation of the globin part of hemoglobin. That other factors are involved, however, in the production of hemoglobin or the stimulus to its production, is suggested by the fact that Whipple, Robscheit-Robbins, and Walden were able to show[2] that a liver fraction other than that most active in the treatment of pernicious anemia was capable of causing new hemoglobin formation. That the effect was not due to the iron content alone was suggested by the observation that there seemed to be "summation response" when this extract and iron were given in combination as contrasted to the administration of either alone.

This liver fraction, called the secondary anemia fraction, or No. 55 fraction, contains that part of the liver material insoluble in 70% alcohol as distinguished from the "pernicious anemia fraction" (No. 343 fraction).

It seemed of interest to determine whether or not there was a possibility that such a fraction might be involved in the augmentation of iron uptake from the gastro-intestinal tract. With the availability of the sensitive means of determining uptake, using iron tagged with the radioactive isotope Fe^{59}, one is provided with a tool with which such an effect could easily be tested. That this "summation effect" is due to an increase in the absorption of iron from the gastro-intestinal tract seems to be pretty well ruled out by the following experiments.

Methods. The subjects studied were 11 normal women, laboratory technicians or secretaries, ranging in age from 21 to 45 years. In addition, 1 patient, who had had repeated massive hemorrhage due to duodenal and gastric ulcers, was included. The subjects were divided into 2 groups, half receiving at the first feeding iron tagged with the radioactive isotope alone and the other half receiving the same dosage level of tagged iron, plus 5 g of liver extract No. 55.† Two weeks after the feeding a single sample of whole blood was drawn into ammonium and potassium oxalate, and this was divided into duplicate samples for estimation of radioactivity content of the red blood cells. At this time those subjects who had received iron alone were then given the same dosage of iron plus the liver extract, and that group which had received both iron and extract were given iron alone. Two weeks later the blood was sampled again, and feeding reverted to the original program as of the first experimental period. In this way each subject acted as his own control.

The radioactive iron was purified as described elsewhere[3] in order to eliminate contamination due to traces of radioactive cobalt, manganese, zinc, nickel, copper, indium, etc. The iron isotope used was a cyclotron

1 Hahn, P. F., and Whipple, G. H., *J. Exp. Med.*, 1939, 69, 315.

2 Whipple, G. H., Robscheit-Robbins, F. S., and Walden, G. B., *Am. J. Med. Sci.*, 1930, 179, 628.

3 Hahn, P. F., *Ind. and Eng. Chem.*, 1945, 17, 45.

TABLE 1.
Effect of X-ray Irradiation on Relative Viscosity, Hyaluronic Acid Concentration, and Total Protein Content of Joint Fluid.

Joint fluid	Time exposed to irradiation (min)	Relative viscosity X H_2O	Total protein, %	Hyaluronic acid (mg per cc)
R.R. of 6/18/46	0	214	4.8	0.24
	2	200	4.9	0.26
	7	151	4.9	0.25
	11	111	4.9	0.24
Pooled	0	22.5	4.9	0.22
	2	19.2	4.9	0.21
	6	16.9	4.9	0.22
	12	12.7	4.9	0.22
R.R. of 3/12/47	0	42.7	5.0	0.16
	3	37.4	5.0	0.16
	8	26.1	5.0	0.16
	14	20.5	5.0	0.16

never as viscous as joint fluid of equal hyaluronic acid concentration[12] has been attributed to the presence of secondary valences in the native material which are broken on purification. The greater susceptibility to irradiation of joint fluid, as compared with that of solutions of hyaluronate, would suggest that these secondary valences may primarily be attacked by the X-rays. The hyaluronic acid in irradiated joint fluid is still susceptible to enzymatic hydrolysis and thus irradiation did not cause a profound change in the hyaluronic acid molecule. Although the amount of irradiation delivered is beyond the physiological range, it is possible, nevertheless, that this effect may be one of the factors which come into play in the response of pathological joints—peripheral and spinal—to irradiation. The widespread presence of the viscous acid mucopolysaccharides of this group throughout the connective tissue of the organism may make this an important factor in the response of many tissues to roentgenotherapy.

Conclusions. 1. The viscosity of joint fluid is decreased by exposure to X-rays, the decrease in viscosity being directly proportional to the amount of irradiation. 2. The viscosity of pure hyaluronic acid solutions is decreased less by the action of X-rays than is the viscosity of joint fluid. 3. Joint fluid is as susceptible to the enzymatic action of bull testis hyaluronidase in the irradiated sample as in the control. No increase in titratable acidity follows irradiation of pure hyaluronate solution, indicating that the reduction in viscosity probably does not result from oxidative degradation. 4. The possible physiological implications are discussed.

in one possibly important matter in that they represented the feeding of single small doses of tagged iron; whereas, the work done on dogs by the other investigators represented daily feedings of iron and liver extract over several weeks periods. Recently Granick[5] has shown that the ferritin of the gastro-intestinal tract increases following the feeding of iron. Thus we must keep in mind the possibility that the body may adapt itself to the absorption of larger amounts of iron through some as yet unknown mechanism for the production of the material which itself is probably concerned with iron absorption.

It is important also to keep in mind the fact that the experimental standard anemic dogs used by Whipple and Robscheit-Robbins in their classical experiments on factors involved in hemoglobin regeneration are not simple "iron deficiency" experimental animals. The iron content of the salmon-bread diet fed these animals is adequate for normal growth and maintenance, but probably not sufficient to provide for the increased demands imposed by the necessity of removal of large amounts of blood occasioned by feedings of active supplements. Since it has been shown, as mentioned earlier,[1] that under certain circumstances restriction of proteins may cause a lowered ability to form sufficient

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[5] Granick, S., *J. Biol. Chem.*, 1946, 164, 737.

hemoglobin in experimental hemorrhagic anemia in dogs, it is possible that under the conditions of the experiments cited by Whipple, Robscheit-Robbins, and Walden that there was a more complicated deficiency involved, in which more than one factor or two interrelating factors were involved.

Whatever the explanation may be as to the different results obtained under these widely various sets of conditions, it is apparent that any influence on hematopoiesis exerted by liver extract No. 55 is not one which acts through its ability to augment the absorption of the iron from the gastro-intestinal tract.

Summary. Iron tagged with the radioactive isotope Fe[59] was fed with and without supplements of liver extract No. 55 to a group of 11 normal adult women. There were no obvious differences in the absorption and utilization of iron when this material was given alone or in conjunction with the "secondary anemia fraction of liver." In one case of human iron deficiency due to multiple acute and chronic hemorrhage there was also no difference in the absorption of iron as indicated by this method.

The "summation response" described by Whipple, Robscheit-Robbins and Walden is apparently not related to the effect of the secondary liver extract on *absorption of iron* in the gastro-intestinal tract.

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The Influence of Oral Saccharin on Blood Sugar.

ERNEST KUN* AND ISTVAN HORVATH. (Introduced by E. M. K. Geiling.)

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The purpose of this investigation was to determine whether or not sweet taste as a nervous sensation has an effect on carbohydrate metabolism. It was found that saccharin, a substance of intensely sweet taste, can cause a decrease in blood sugar, presum-

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ably through the reflex liberation of insulin.

Methods. Saccharin (Sodium salt o-benzoic-acid-sulfimide) was given to human subjects in doses of 0.05 g in 80 ml water. In one group of experiments the effect of single doses, and in another group the effect of repeated doses of saccharin solution was followed.

TABLE I

Effect of Concomitant Feeding of Secondary Liver Extract (No. 55 Fraction) on Absorption of Iron Tagged with the Radioactive Isotope Fe59. Dose of Fe = 39 mg.*

Subject	Wt kgm	RBC Hct.%	Uptake of tagged iron in % of administered dose when fed			
			Fe Alone	Fe + Liver	Fe Alone	Fe + Liver
E. C.	48.6	43	9.1	7.8	7.9	
P. J.	48.2	42		4.1	6.4	0.7
B. R.	53.5	44	1.4	1.4	3.9	
M. C.	50.4	44		5.3	9.9	7.1
D. R.	49.2	38	5.0	3.0	3.7	
J. C.	44.6	41		2.1	1.7	1.2
I. L.	39.7	43	5.1	1.2	4.2	
J. A.	55.8	44		1.5	7.4	2.3
M. G.	58.9	41	3.6	1.1	3.6	
B. P.	57.6	43		8.9	9.8	6.8
H. T.	52.2	42			4.7	7.8
C. P.	73.6	22	49.0	47.0		

* Except Subject C.P. who received 25 mg.

prepared product, Fe59, made by the d-p reaction on Fe58. The blood withdrawn was centrifuged in graduated 15 ml tubes in a type 1 International standard 8 unit head for 35 minutes at 2800 r.p.m. or more. The plasma was discarded following reading of the hematocrit value, and the red cells washed into Pyrex beakers, dried, ashed in a muffle furnace at 625°C, and the resulting iron electroplated as described elsewhere.[3] Radioactivity measurements were made, using a thin mica window, argon filled bell type Geiger counting tube, in conjunction with M.I.T. counting rate meter. The assumption was made that all iron absorbed was utilized, and the calculation of uptake was based on calculations described previously.[4]

Experimental Observations. In Table I, below, are summarized the results of the experiments carried out. The iron uptakes as measured with the radioactive tracer varied in these women from 1½ to 10% of the administered dose. The series involved is too small to allow one to speculate effectively on the meaning of such variation. It is to be noted, however, that with such a wide range of uptake, one must interpret carefully the absorption of single doses of iron in attempting to use this technique for diagnostic purposes.[3] It was for this reason that the pro-

cedure was carried out as described above, where each individual would furnish a control for each particular experiment. It is obvious on inspection of Table I that there is no enhancement of the uptake of the tagged iron resulting from the concomitant administration of the secondary liver extract. If anything, there is a suggestion that the liver extract might conceivably, to a small extent, inhibit the absorption of iron, although this may not be statistically significant. The rationale of such an inhibition might be related to the presence of phosphates or related material, which act to precipitate iron in the gastro-intestinal tract and prevent its absorption before it has passed beyond the stomach and duodenum.

Discussion. It is of interest to note that the one individual who had a marked iron deficiency type of anemia related to repeated acute and chronic blood loss, patient C.P. as shown in the table, showed the typical high efficiency of absorption and utilization of iron which one associates with such a condition, and this contrasts very markedly with the uptakes in the normal laboratory workers and secretaries who made up the remainder of the experimental group of subjects.

It is necessary to point out that these experiments, in spite of the sensitivity of quantitation attainable through the use of the radioactive isotopes over the technique used by Whipple and Robscheit-Robbins, differed

4 Hahn, P. F., Bale, W. F., Ross, J. F., Balfour, W. M., and Whipple, G. H., *J. Exp. Med.,* 1943, 78, 169.

TABLE II

Blood sugar values determined at 15-minute-intervals in individuals under normal fasting conditions (F) and in the same individuals under the same conditions after oral administration of 0.05 gm saccharin in 80 ml water, given in 4 equally divided doses at 10 minute intervals after 0 time. Blood sugar values are expressed in the same way as in Table I.

Indiv. Subj. No.	15 min		30 min		45 min		60 min		75 min		90 min		105 min	
	F	S	F	S	F	S	F	S	F	S	F	S	F	S
1.	98.0	100.0	101.2	87.2	100.0	81.2	96.2	91.0	95.4	93.6	99.3	106.1	98.3	104.2
2.	96.0	86.0	104.4	84.3	96.2	84.0	95.3	90.2	98.0	85.4	105.3	97.6	105.1	102.1
3.	101.0	88.0	96.0	86.2	98.5	85.7	101.0	81.6	96.2	90.0	103.7	92.1	102.0	106.1
4.	97.0	97.1	100.0	89.2	102.2	82.0	103.0	83.7	101.0	87.3	96.7	106.0	100.1	104.2
5.	98.0	90.7	97.4	87.0	97.1	86.3	102.7	80.0	97.3	91.1	102.0	95.5	97.3	97.3
6.	99.2	90.6	98.8	84.0	99.0	82.5	100.4	85.3	98.0	88.3	104.1	106.1	99.3	96.2
	M=98.3	M=91.0	M=99.6	M=86.3	M=98.8	M=83.5	M=99.6	M=84.0	M=97.7	M=89.2	M=101.8	M=100.5	M=100.3	M=100.1
	$t = 5.6$		$t = 7.7$		$t = 10.4$		$t = 6.5$		$t = 6.0$		$t = 0.4$		$t = 0.1$	

and the liver. Althausen and Wewer[6] could not confirm this observation. These authors disregarded the relatively small but reproducible decrease in blood sugar after saccharin consumption, although according to their results this decrease was observed. Fischer and Schroter[7] investigated the effect of saccharin on human patients suffering from various diseases, but no control tests on healthy subjects were carried out. Some of these patients showed a decrease in blood sugar, but the authors attached no significance to this phenomenon. The small but significant decrease in blood sugar level can be seen only under circumstances in which all other influences are eliminated or controlled in every way possible. As an explanation of the observed phenomenon, it seems logical to think of insulin liberation, called forth by a nervous impulse through the sensory fibers from the taste endplates to the vagus centers. Since Geiger's experiments[8] showed that the vagus is a secretory nerve of the islets of Langerhans, it is possible that the efferent part of this reflex arc is in the vagus. Other afferent stimuli from sensory organs, such as the olfactory, cause a similar decrease in blood sugar, as reported by Bassi and Pascucci,[9] whose observation also strengthens the possibility of the existence of the above described reflex mechanism.

Summary. 1. Oral administration of an aqueous solution containing 0.05 g of saccharin caused a decrease of 12 to 16% in the blood sugar level of normal individuals. Water alone had no significant influence.

2. The decrease of blood sugar was not proportional to the saccharin dosage.

3. Administration of the saccharin in 4 portions during a 40 minute period increased the duration of hypoglycemia.

4. It is suggested that this phenomenon is due to the influence of the sweet taste, which may act by means of a reflex mechanism to induce insulin secretion.

6 Althausen, T. L., and Wewer, G. K., Proc. Soc. Exp. Biol. and Med., 1937, 35, 517.

7 Fischer, F., and Schroter, A., Dtsch. Med. Wschr., 1935, 61, 1354.

8 Geiger, E., Arch. Exp. Path. and Pharmacol., 1928, 134, 317.

9 Bassi, M., and Pascucci, P., Rass. Neur. Veget., 1943, 3, 68.

TABLE I

Blood sugar values, determined at 10 minute intervals in individuals under normal fasting condition (F) and in the same individuals under the same conditions after oral administration of 0.05 gm saccharin in 80 ml water (S). Blood sugar values are expressed in terms of percentage of the blood sugar level at 0 time.

Indiv. Subj. No.	10 min		20 min		30 min		40 min		50 min	
	F	S	F	S	F	S	F	S	F	S
1.	96.2	98.4	104.2	90.0	98.1	84.3	102.1	93.4	98.8	90.1
2.	98.1	100.1	102.4	91.4	97.3	85.2	96.4	87.6	96.2	98.3
3.	97.0	96.2	100.1	93.2	96.4	88.7	100.1	88.2	103.2	104.1
4.	96.6	98.8	98.4	85.1	101.1	90.3	95.4	95.1	99.3	99.1
5.	101.4	99.3	97.3	80.3	95.2	80.2	98.3	85.3	100.1	97.5
6.	97.6	102.4	96.4	90.1	93.4	78.1	97.6	81.1	98.8	93.6
7.	104.2	101.3	101.3	83.1	95.3	84.1	99.4	92.1	92.3	97.2
8.	98.9	97.7	100.2	92.2	96.0	85.3	96.2	98.6	100.0	106.9
9.	100.1	99.8	97.0	95.4	97.1	82.0	97.0	99.1	95.4	94.2
	M=98.8	M=99.3	M=99.6	M=88.8	M=96.6	M=84.2	M=98.0	M=91.2	M=98.2	M=97.9
	t = 0.5		t = 5.7		t = 7.7		t = 3.2		t = 0.15	

In the first group 9 persons were used. In the morning, before eating, the subjects remained in bed, and blood sugar determinations were carried out at 10 minute intervals for 50 minutes. Blood was taken from the cubital vein and blood sugar determinations were done with 0.1 ml samples by an iodometric titration method.[1] A fasting level was thus established. Each subject was then used at the same time the next day under the same conditions for the saccharin experiment. Ten minutes after the first blood sample was taken, the subject drank a solution of saccharin (0.05 g dissolved in 80 ml of water). As standard procedure the drinking lasted for 5 minutes.

Results. In 5 control experiments 80 ml water had no significant effect on the blood sugar. The results are given in tables where the blood sugar values are expressed in terms of per cent of the initial blood sugar level. This initial level was arbitrarily taken as 100. In Table I are shown the changes in blood sugar level following a single saccharin administration. The means of 9 parallel experiments were compared with the means of 9 controls. The significance of the difference between the means was tested by calculating the "t" values according to Fisher.[2] Following administration of the saccharin solution

a rapid decrease in blood sugar occurred which ended after 30 minutes.

The second group of 6 persons was given the same amount of saccharin solution as was given to Group I, but it was divided into 4 parts which were drunk at 10 minute intervals. Blood samples were drawn at 15 minute intervals for 90 minutes. The administration of saccharin solution in 4 parts resulted in the same drop in blood sugar, except that the hypoglycemia lasted 75 minutes. The results of this experiment are shown in Table II.

Discussion. A comparison of the 2 series of experiments reveals no quantitative correlation between the decrease in blood sugar and the amount of saccharin taken. Salkowski,[3] and Carlson, Eldridge and Foran[4] showed that saccharin has no pharmacological activity in the small doses used here. In both experiments, the sensation of intense sweet taste was produced and there is good reason to believe that this taste, and not the amount of saccharin was responsible for the blood sugar effect. It would be of interest to test other sweet tasting substances for this effect. Syllaba[5] reported an increase in blood sugar after the administration of saccharin by means of a gastric tube in rabbits and man. He suggested that this effect is due to a reflex mechanism between the intestinal wall

1 Fujita, A., and Iwatake, D., *Biochem. Z.*, 1931, 242, 43.

2 Fisher, R. A., *Statistical Methods for Research Workers*, 1930, Oliver and Boyd, London.

3 Salkowski, A., *Virchows Arch.*, 1886, 105, 46.

4 Carlson, J., Eldridge, and Foran, *J. Metabol. Res.*, 1929, 8, 451.

5 Syllaba, G., *Am. J. Physiol.*, 1929, 90, 535.
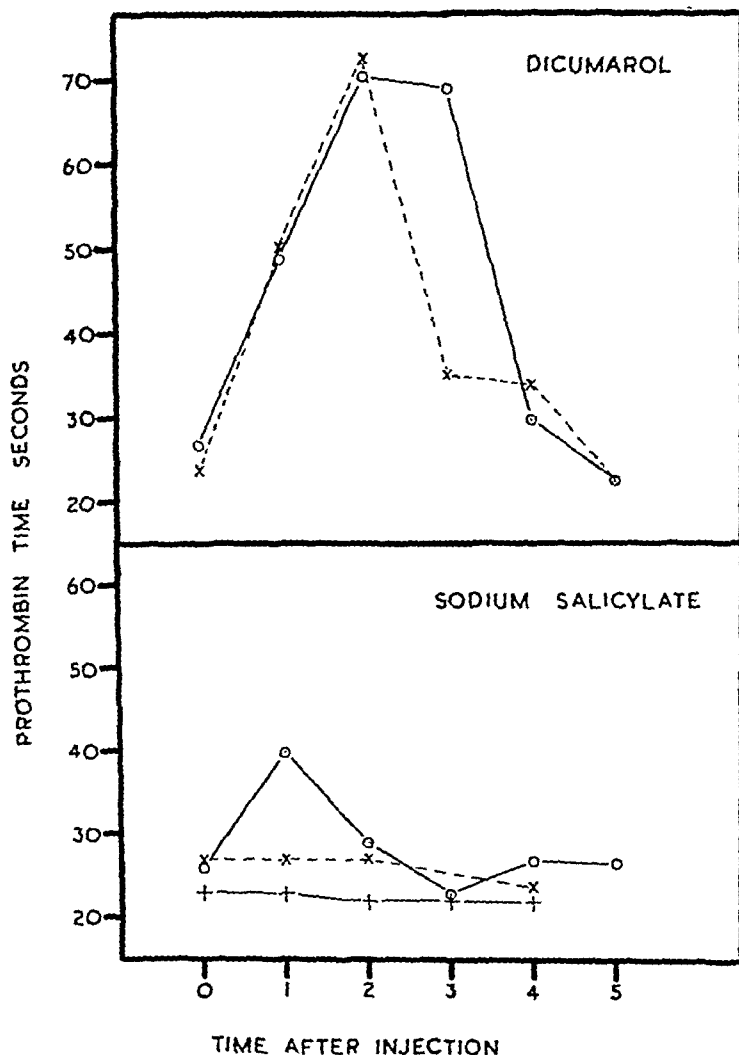
FIG. 1.
Effect of sodium salicylate and dicumarol on prothrombin time in rabbits. Rabbit 16. 6 mg of dicumarol, 0.8 g of sodium salicylate. —O—O— Oral; ——×——×—— Intravenous; —+—+— 1 g of sodium salicylate + 2 g of sodium sulfasuccidine orally.

control). The prothrombin response of each rabbit to 6 mg of dicumarol and 0.5 g/kg of sodium salicylate was determined both on oral and intravenous administration. The response was also determined after the oral administration of 2 g of sodium sulfasuccidine and of 2 g of sodium sulfasuccidine plus 0.5 g kg of sodium salicylate. The sodium sulfasuccidine was given in 4 divided doses at 9 a.m. and 4 p.m. on 2 successive days, the sodium salicylate being given at 2 p.m. of the second day. After the experimental period of 5 to 6 days, a period of 4 days to several weeks was allowed before testing the next dose. The responsiveness of the animals was checked at intervals in the course of the experiment with the standard dose of dicumarol intravenously. A similar series was attempted using acetylsalicylic acid but it was found that the dose required orally to affect the prothrombin time was above the lethal dose when given intravenously.

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Action of Sodium Salicylate on Prothrombin Time in Rabbits.[*]

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Link[1] has postulated, on the basis of the degradation of dicumarol to salicylic acid *in vitro*, that the hypoprothrombinemic action of dicumarol is due to its degradation to salicylic acid *in vivo*. Supporting this hypothesis was the finding of Link, Overman, Sullivan, Huebner, and Scheel[2] that single doses of salicylic acid administered either orally or intravenously to rats maintained on an artificial diet low in vitamin K, caused a temporary hypoprothrombinemia. The increase in prothrombin time after administration of salicylates has been confirmed clinically by Rapoport, Wing and Guest,[3] Shapiro[4] and others. However, Link's views on the relationship of the action of dicumarol and salicylate on prothrombin have not been supported. Link's data indicate that salicylates have a very weak action in lowering prothrombin compared with dicumarol. Lester[5] failed to find salicylates in the urine after the administration of dicumarol to rats. Stefanini and Petrillo[6] observed that the addition of sodium salicylate to human plasma *in vitro* in concentrations greater than 0.2% markedly lengthened the prothrombin time. Clark and Spitalny[7] have observed that other analgesic-antipyretic drugs (antipyrine, aminopyrine, acetanilid, acetophenetidin and cinchophen)

have a similar action on prothrombin time, and that the prothrombinopenic action of salicylate was greatly augmented by hyperthermia and by an increased metabolism from other causes. This suggests that an important contributing factor to the action of salicylates on prothrombin clinically is the accompanying clinical syndrome, and that the effect observed by Link and co-workers is a nonspecific effect, unrelated to the action of dicumarol.

Since dicumarol has been synthesized from salicylic acid and since dicumarol is presumably formed from the coumarins in spoiled sweet clover by bacterial action,[1] there remains as a further possible basis for the prothrombopenic action of salicylates, the conversion of these substances to dicumarol or related compounds. To test this possibility, a study has been made of the response to dicumarol and sodium salicylate in rabbits upon oral and intravenous administration.

Methods. Normal rabbits of 2 kg body weight and maintained on the normal colony diet were used. Prothrombin times were determined daily, on both undiluted plasma and also on 50, 25, 12.5 and 6.25% plasma. As reported by previous workers, the salicylate had little effect on the prothrombin time of undiluted plasma so that all the results reported are for the 12.5% plasma. Prothrombin times were determined by the Quick technique, using an acetone-dried horse brain powder for preparation of the thromboplastin.[8] We are indebted to Dr. L. A. Kazal of Sharp and Dohme, Inc., who kindly supplied this material. To control minor variations in thromboplastin, in all cases a normal rabbit with the same initial prothrombin time was included in each series and the prothrombin times are reported as experimental/(normal

1 Link, K. P., *Harvey Lecture Series*, 1943-4, 39, 162.

2 Link, K. P., Overman, R. S., Sullivan, W. R., Huebner, C. F., and Scheel, L. D., *J. Biol. Chem.*, 1943, 147, 463.

3 Rapoport, S., Wing, M., and Guest, G., Proc. Soc. Exp. Biol. and Med., 1943, 53, 40.

4 Shapiro, S., *J. Am. Med. Assn.*, 1944, 125, 546.

5 Lester, D., *J. Biol. Chem.*, 1944, 154, 395.

6 Stefanini, M., and Petrillo, E., *Boll. Soc. Ital. Biol. Sper.*, 1946, 22, 366.

7 Clark, B. B., and Spitalny, M., *Fed. Proc.*, 1946, 5, 171.

8 Kazal, L. A., Higashi, A., and Arnow, L. E., Proc. Soc. Exp. Biol. and Med., 1945, 60, 196.

time of 12.5% plasma, of oral and intravenous administration of sodium salicylate, and of dicumarol has been compared in rabbits on a normal diet.

The oral and intravenous administration of dicumarol gave the same prolongation of the prothrombin time. However, while the oral administration of 0.5 g/kg of sodium salicylate definitely prolonged the prothrombin time, the intravenous administration of the same dose in the same animal did not result in any change in the prothrombin time. After the oral administration of sodium sulfasuccidine, the oral administration of sodium salicylate did not affect the prothrombin time.

It is suggested as an explanation of these results that salicylate may be converted to dicumarol or a substance with similar prothrombinopenic properties by bacterial action in the intestinal tract.

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Quantitative Aspects of the Inhibition of Anaphylactic Shock in Guinea Pigs.

STANLEY MARCUS. (Introduced by Walter J. Nungester.)

From the Rackham Arthritis Research Unit, *Medical School, University of Michigan, Ann Arbor.*

While the modifying action of the proprietary compounds β-dimethylaminoethyl benzhydryl ether hydrochloride (benadryl) and N'-pyridil-N'-benzyl-N-dimethylethylenediamine (Pyribenzamine) on histamine and anaphylactic shock has been vigorously investigated,[1] some conflicting data have appeared in the literature with regard to the action of these drugs on true anaphylactic shock in guinea pigs. Mayer, *et al.*,[2] demonstrated that 5 guinea pigs injected with horse serum 21 days before, were protected against an intracardial shock dose of 0.5 cc of horse serum when they were previously treated subcutaneously with 1.0 mg/kg of pyribenzamine. Campbell, *et al.*,[3] reported that although benadryl offered effective protection against histamine shock in the rabbit, it was in-

effective in controlling anaphylaxis in actively sensitized (hen egg white) rabbits and guinea pigs. These latter workers used shock doses of 0.75 cc of the antigen for guinea pigs, given intraperitoneally. They conclude that their results seem at direct variance with those of Loew and Kaiser[4] and of Friedländer, *et al.*,[5] since these groups had found, similarly to Mayer's group,[2] that benadryl offers marked protection against anaphylaxis in the guinea pig.

Since in none of this previous work on benadryl and pyribenzamine cited was the attempt made to put protection against anaphylaxis in the actively sensitized animal on a quantitative basis, similar experiments were repeated with this view in mind.

Materials and Methods. The whites of 3 hen eggs were separated from the yolks, strained through cheesecloth into a beaker and then stored in a rubber stoppered vaccine bottle in the refrigerator. This material (Pro-

* The Rackham Arthritis Research Unit is supported by the Horace H. Rackham School of Graduate Studies of the University of Michigan.

1 Feinberg, S. M., *J. A. M. A.*, 1946, 132, 702.

2 Mayer, R. L., Huttrer, C. P., and Scholz, C. R., *Science*, 1945, 102, 93.

3 Campbell, B., Baronofsky, I. D., and Good, R. A., PROC. SOC. EXP. BIOL. AND MED., 1947, 64, 281.

4 Loew, E. K., and Kaiser, M. E., PROC. SOC. EXP. BIOL. AND MED., 1945, 58, 235.

5 Friedländer, S., Feinberg, S., and Feinberg, A. R., PROC. SOC. EXP. BIOL. AND MED., 1946, 62, 65.

	Salicylate			Dicumarol	
Rabbit	Oral	Intrav.	+ sulfa-succidine.	Oral	Intrav.
6	31.0	26.8		69.2	71.4
	(26.5)	(26.7)		(24.8)	(26.3)
11	26.8	25.2	22.4	45.0	65.0
	(25.0)	(26.3)	(21.8)	(24.8)	(26.2)
12	27.0	26.2	22.0	70.2	72.3
	(24.8)	(26.3)	(21.8)	(25.9)	(22.6)
13	31.0	26.5		32.0	47.5
	(27.2)	(26.4)		(25.0)	(26.3)
16	40.0	27.8	23.0	70.2	72.3
	(27.2)	(27.3)	(21.8)	(25.9)	(22.6)

( ) = control prothrombin time on normal rabbit.

Results and Discussion. The prothrombin time response to the oral and intravenous administration of dicumarol and of sodium salicylate and to the oral administration of sodium salicylate + sodium sulfasuccidine are shown in Fig. 1. The peak prothrombin times obtained in 5 rabbits with these substances are reported in Table I. A definite increase in prothrombin time was observed after the oral administration of sodium salicylate. However, with the same dose administered to the same rabbit intravenously, no change in the prothrombin time was observed, although as previously reported by many investigators, the prothrombin response to dicumarol given intravenously to the same animal was of the same extent as after oral administration. Since Vitamin K was not withheld from the diet, the oral administration of sodium sulfasuccidine did not affect the prothrombin time. However, as shown in Table I, it did prevent the increase in prothrombin time after the administration of sodium salicylate.

The most direct explanation of the difference in the results on oral and intravenous administration of sodium salicylate is conversion of salicylate to dicumarol or a similarly acting compound in the intestinal tract. The action of sodium sulfasuccidine in abolishing the prothrombopenic action of salicylate suggests that this conversion is due to bacterial action. Attempts have been made to demonstrate such conversion with intestinal contents *in vitro*, by isolation of the products.

This was unsuccessful. It may have been due to adverse conditions for bacterial synthesis or due to the small amounts of material involved. The prothrombopenic action of 1 g of sodium salicylate in the rabbits was equivalent to that of approximately 2 mg of dicumarol, so assuming formation of this compound, less than one per cent of the salicylate underwent conversion.

These results are somewhat at variance with those of Link[1] who reported that intravenous administration of sodium salicylate to rats did cause an increase in prothrombin time. This difference in results may be due to the use of a different animal species and also may be related to the vitamin K deficiency produced in the rats by Link. Further, Link does not give direct comparisons of oral and intravenous administration and his results are the average response of 6 rats. As originally shown by Link[1] on rabbits, marked differences occur in the response of individual animals to dicumarol (and also salicylate, Table I), and this is accentuated on a vitamin K-deficient diet. The results of Clark and Spitalny, of Stefanini and Petrillo, and the results reported here suggest that there are 3 possible mechanisms operative in the increased prothrombin time after the administration of salicylates, namely, a general action related to their analgesic-antipyretic properties, a direct effect on the clotting system of the blood, and lastly, conversion in the intestinal tract to dicumarol or a similarly acting compound.

Summary. The effect on the prothrombin

TABLE II
Effect of Pyribenzamine on Amount of Egg White Required to Shock Actively Sensitized Guinea Pigs (300–400 g) by Intracardial Injection.

| Animal No. | Days after Sensitization | Antigen Dilution ml | No. of Doses LD_{100} | Premedication | | Result* |
				Intraperitoneal Dose mg	Minutes Shocking Before	
1	19	.1 of 1:4	1	5	10	0
2	19	.4 1:4	4	5	15	0
3	19	.4 1:4	4	5	20	0
4	19	.8 1:4	8	5	15	2
5	19	.8 1:4	8	5	15	4
6	19	.4 1:2	8	5	15	1
7	19	.4 1:2	8	10	15	4
8	19	.8 1:4	8	10	20	1
9	19	.4 1:2	8	10	20	1
10	19	.5 1:2	10	5	30	3
11	19	.6 1:2	12	10	15	3
12	19	1.0 1:2	20	5	20	3
13	20	.6 1:4	6	10	30	0
14	20	.8 1:4	8	10	15	1
15	20	.4 1:2	8	10	20	1

*Code same as that used in Table I.

TABLE III
Effect of Benadryl on Amount of Egg White Required to Shock Actively Sensitized Guinea Pigs (300–400 g) by Intracardial Injection.

| Animal No. | Days after Sensitization | Antigen Dilution ml | No. of LD_{100} Doses | Premedication | | Result* |
				Intraperitoneal Dose mg	Minutes Before Shocking	
1	20	.1 of 1:4	1	5	20	0
2	20	.4 1:4	4	5	25	0
3	20	.5 1:2	10	5	35	3
4	21	.4 1:4	4	10	20	0
5	21	.6 1:4	6	10	20	0
6	21	.4 1:2	8	5	15	2
7	21	.4 1:2	8	10	25	1
8	21	.4 1:2	8	10	30	1
9	21	.4 1:2	8	10	15	1
10	21	.5 1:2	10	10	20	2
11	21	.5 1:2	10	10	25	3
12	21	.6 1:2	12	5	25	3
13	21	.6 1:2	12	5	25	3
14	21	.6 1:2	12	10	25	3

*Code same as that used in Table I.

which received more than 8 L.D.$_{100}$ doses (i. e., 12 or 10) all died within 7 minutes, exactly as had the control animals. Four animals receiving less than 8 L.D.$_{100}$ doses (1, 4, 6) exhibited no symptoms of shock.

Summary and Conclusions. The L. D.$_{100}$ dose of a solution of hen egg white by intracardial injection was determined for a group of actively sensitized guinea pigs. It was found that 5 to 10 mg of pyribenzamine or benadryl injected intraperitoneally between 10 and 30 minutes before giving the shocking dose protected the animals against approx-

imately 8 L. D.$_{100}$ doses of the antigen while injection of 10 or more L. D.$_{100}$ doses resulted in rapid death with symptoms indistinguishable from the control animals. If 6 or less L. D.$_{100}$ doses were given, no symptoms were noted in the protected group.

These results seem to indicate that the so-called anti-histaminic drugs do exert a protective influence, quantitative in nature, against anaphylaxis in the actively sensitized guinea pig. This fact might well be expected since a similar quantitative protection is obtained with these drugs against histamine

tein $= 11.53$ g/%; NPN $= 21.66$ mg/%†)
served as the source of sensitizing and shock-
ing antigen throughout these experiments.
The guinea pigs used weighed between 300
and 400 g. They received sensitizing injections
of 0.1 and 0.2 ml of the egg white intra-
peritoneally and were kept in large common
cages until ready for use. Intracardial in-
jections were made using tuberculin syringes
with No. 20 or 21, one or one and one-half
inch needles. No anesthetic was employed.
After proving the presence of the hypodermic
needle in the heart by withdrawing 0.2 to
0.4 cc of blood, the egg white and blood mix-
ture was reinjected slowly, within 30 seconds.

Dilutions of the egg white were made after
centrifuging 3 to 4 cc of the refrigerated stock
supply. The dilutions were made in saline with
1.0 ml serological pipettes graduated in hun-
dredths and were in the range of the probable
lethal dose (L. D.$_{100}$) as determined by earlier
experience.

The pyribenzamine and benadryl used were
the commercial preparations put up for oral

TABLE I
Determination of LD$_{100}$ and Maximum Non-
Lethal Dose of Egg White Required to Shock Ac-
tively Sensitized Guinea Pigs (19–21 days; 300–
400 g) by Intracardial Injection.

No. of Animals	Antigen Dilution ml	Result*
1	.1 of 1:16	0
8	.1 1:8	1, 1, 1, 1, 1, 1, 1, 2
3	.2 1:8	3, 3, 3
6	.1 1:4	3, 3, 3, 3, 3, 3

* Results of intracardial injection were num-
   bered as follows:
0, no symptoms noted.
1, mild symptoms. Face washing, coughing or
   sneezing, animal survives.
2, more severe symptoms, strong inspiratory
   movements, incontinence, animal survives.
3, severe symptoms and death within 3–8 minutes.
   Massive emphysema of lungs at autopsy.
4, severe symptoms, prostration followed by death
   after a delayed period of 15 to 60 minutes or
   more. Lungs not markedly emphysemic, but
   show some congestion. Congestion of kidneys,
   adrenals and mesenteric blood vessels. Beet-red
   appearance of stomach and intestinal outer
   wall. Lining of peritoneum dark pink to red.

† Nitrogen determinations (Kjeldahl) were made
after concluding the experiments. The egg white
was inadvertently frozen before the nitrogen deter-
minations were made.

use and were incompletely soluble in 0.9%
sodium chloride solution. These preparations
were kept in test tubes and shaken to insure
homogeneous suspension before placing in a
syringe for intraperitoneal injection.

Results. Table I illustrates a typical titra-
tion to determine the L. D.$_{100}$ of the egg white
antigen used in these experiments. Among this
group of animals sensitized 19, 20, or 21 days
previously, none survived the intracardial in-
jection of 0.1 ml of a 1:4 dilution or its con-
centration equivalent and none died of the
effects of 0.1 ml of a 1:8 or higher dilution.
The conclusion reached was that 0.1 ml of a
1:4 dilution represented an L. D.$_{100}$ dose.

Table II shows the results obtained when
sensitized guinea pigs, protected by pyribenza-
mine were injected with the same dilutions
of egg white on the same day that the control
animals were injected. These animals were
injected intraperitoneally with the pyribenza-
mine 10 to 30 minutes preceding the shocking
dose of egg white. They received either 5 or
10 mg of the drug with no attempt being made
to vary the drug on a weight basis. Among 8
guinea pigs receiving 8 L. D.$_{100}$ doses of egg
white, 2 died after a delayed period and
necropsy showed what appeared to be the
systemic result of anaphylaxis in the guinea
pig. The other 6 animals exhibited mild to
severe symptoms, but lived in each case.
Among 4 animals receiving less than 8 L. D.$_{100}$
doses (*i. e.,* 1, 4, and 6) none exhibited symp-
toms of any type. Among 3 animals re-
ceiving more than 8 L. D.$_{100}$ doses (*i. e.,*
20, 12, 10) all died within 7 minutes, in
every way similarly to the control animals.

*Table III illustrates the results obtained
with sensitized guinea pigs protected against
shock by benadryl which was intraperiton-
eally injected 15 to 30 minutes preceding the
dose of egg white. Again, 5 or 10 mg of the
drug was given with no effort being made to
vary dosage according to weight of the animal.
Among 4 animals receiving 8 L. D.$_{100}$ doses
none died although all exhibited mild to
severe symptoms. One animal injected with
10 L. D.$_{100}$ doses 20 minutes after receiving
10 mg of benadryl had violent symptoms but
survived the shock and lived. Five others*

onated eggs inoculated into the allantoic cavity with certain members of this group. Consequently, this method of cultivation was utilized in the preparation of agents used as vaccines in the present work. Infected allantoic fluids were pooled, filtered through sterile gauze pads and stored in the dry ice-box until needed. Immediately prior to irradiation, the frozen materials were thawed at 37°C and filtered again to remove the small amount of insoluble flocculent precipitate frequently present.

In order to determine the least amount of irradiation necessary to inactivate the agents completely, an allantoic fluid preparation of each virus was divided into several portions which were irradiated for different periods of time. The exposure periods employed varied by 0.05-0.10 seconds and ranged from 0.05-0.30 seconds. It was found that periods of 0.10, 0.15, and 0.20 seconds were required to inactivate our preparations of psittacosis, human pneumonitis, and ornithosis viruses, respectively. A larger quantity of each virus was then titrated by intracerebral inoculation of mice, and inactivated by exposure to ultraviolet irradiation for the minimal time necessary as determined by the preliminary trials. Complete inactivation was confirmed in each instance by the tests described below. The LD_{50} titers,[13] by intracerebral test, before irradiation of the preparations finally used for immunization, were 0.03 cc x $10^{-6.3}$, $10^{-6.5}$, and $10^{-5.8}$, respectively, for the viruses of psittacosis, human pneumonitis, and ornithosis. Inactivated virus was stored in the dry ice-box, without the addition of a preservative, until used for the immunization of mice.

Rigorous tests for complete inactivation of irradiated virus were employed. They consisted of 3 serial intracerebral passages in mice and 3 serial allantoic passages in embryonated eggs. If these passages were all negative for active virus, 3 serial passages were performed by the intranasal route in mice and by the yolk sac route in embryonated eggs. In every instance, when active virus was present, as in the preliminary trials,

it was demonstrated in either the first or second serial intracerebral or allantoic passage. Evidence suggested that the intracerebral route in mice and allantoic route in eggs were equally sensitive in the detection of active material.

Mice were vaccinated intraperitoneally with 2 and 3 0.5 cc injections of undiluted irradiated virus given at 5- or 7-day intervals. The challenge dose of homologous virus consisted of suspensions of infected mouse brain or pooled liver and spleen tissue, and was given either intracerebrally or intraperitoneally to groups of mice together with controls, 3 weeks after the terminal injection of vaccine. The LD_{50} titers of these suspensions were determined before use by intracerebral or intraperitoneal inoculation of mice, depending upon the tissue source.

The emulsion of mouse brain passage virus, first used for intraperitoneal challenge of mice (those which received 2 injections of psittacosis vaccine, Table I) was not sufficiently virulent by this route to give regular, clear-cut results. An attempt was made to overcome this circumstance by performing several rapid serial intraperitoneal passages with emulsions of pooled virus-infected liver and spleen tissue. A definite enhancement in virulence was obtained after 8 passages as indicated by a decrease from 7 to 2 days in the average survival time of animals inoculated with 10% emulsions. An emulsion of pooled infected liver and spleen tissue was then prepared, titrated, and used as the challenge inoculum in the experiment where 3 injections of psittacosis vaccine were employed.

Results. The results shown in Tables I and II indicate that a definite degree of protection was demonstrable in most experiments. Little or no resistance was seen in mice challenged intracerebrally with psittacosis virus (Table I), but definite immunity was demonstrated in psittacosis-vaccinated mice when the challenge dose was given intraperitoneally. Evidently this resistance was not due in part to non-specific immunity since no protection was seen in mice which had received three intraperitoneal injections of normal allantoic fluid three weeks before the challenge dose of

13 Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, 27, 493.

shock.[1,2,3] The modifying action of these drugs again seems in accord with the theory that histamine plays a major role in anaphylaxis. It is of interest that approximately the same degree of protection was offered, in these experiments, by both the compounds used.

16028

Immunization of Mice Against Viruses of the Psittacosis Group with Ultraviolet-Inactivated Vaccines.*

ROBERT D. FRANCIS, ALBERT MILZER, AND F. B. GORDON.

From the Department of Bacteriology and Parasitology, The University of Chicago, and the Samuel Deutsch Serum Center, Michael Reese Hospital, Chicago.

Various workers have demonstrated[1-4] that formalinized suspensions of psittacosis virus possess antigenicity as judged by their capacity to elicit active immunity in mice and avian species. No report has appeared, to our knowledge, of the effect of ultraviolet rays on this or other viruses of the psittacosis group. Levinson, Milzer, and co-workers, employing a new method of inactivating turbid suspensions of viruses and bacteria by ultraviolet rays, have found that it is possible to prepare experimental vaccines[5-7] with dysentery bacilli, and rabies, St. Louis encephalitis, and poliomyelitis viruses with a minimal loss of antigenicity. Briefly, this technic consists of exposing a continuously flowing thin film of suspension to the Oppenheimer-Levinson type lamp which is a powerful source of total and extreme (below 2000 Angstroms) ultraviolet energy. This method of inactivation was employed in the present study with the viruses of psittacosis (6BC), human pneumonitis (SF), and ornithosis (207).

Previous experiments[8] have indicated that these viruses can be completely inactivated by this method and still retain antigenicity as determined by the production of neutralizing antibody in chickens repeatedly inoculated with these preparations. It was our purpose here to make a preliminary investigation of the possibility of using such preparations for the induction of resistance against challenge injections of active virus.

Materials and Methods. The strains of virus employed have been used in previous work in this laboratory and their sources have been recorded elsewhere.[9] Earlier studies[10-12] have shown that large amounts of virus in relatively clear suspensions can be obtained by harvesting the allantoic fluid from embry-

* This investigation has been supported in part by the Commission on Influenza, Board for Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Office of the Surgeon General, U. S. Army; and the John Rockefeller McCormick Memorial Fund of the University of Chicago.

1 Bedson, S. P., *Brit. J. Exp. Path.*, 1938, **19**, 353.

2 Yanamura, H. Y., and Meyer, K. F., *J. Immunol.*, 1942, **44**, 195.

3 Meyer, K. F., Eddie, B., and Yanamura, H., *J. Immunol.*, 1942, **44**, 211.

4 Morgan, H. R., and Wiseman, R. W., *J. Infect. Dis.*, 1946, **79**, 131.

5 Shaughnessy, H. J., Milzer, A., Neal, J., and Levinson, S. O., *J. Infect. Dis.*, 1946, **78**, 69.

6 Levinson, S. O., Milzer, A., Shaughnessy, H. J., Neal, J. L., and Oppenheimer, F., *J. Immunol.*, 1945, **50**, 317.

7 Milzer, A., Oppenheimer, F., and Levinson, S. O., *J. Immunol.*, 1945, **50**, 331.

8 Francis, R. D., to be published.

9 Hilleman, M. R., *J. Infect. Dis.*, 1945, **76**, 96.

10 Eaton, M. D., Martin, W. P., and Beck, M. D., *J. Exp. Med.*, 1942, **75**, 21.

11 Williams, S. E., *Aust. J. Exp. Biol. and Med. Sci.*, 1944, **22**, 205.

12 Francis, R. D., and Gordon, F. B., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 270.

cloth recommended by Weigl. The caged lice are fed twice daily on a healthy nourisher whose skin is first sterilized with 70% alcohol. Between feedings the cages are kept in sterile paper boxes at 34°C. Under these circumstances, masses of eggs are laid on the cloth. These eggs are removed and washed first with 30% alcohol or other suitable weak disinfectant and then with sterile saline, bacterial sterility of the washed eggs being checked by culturing the last drops of the saline wash on nutrient agar. The sterile eggs are dried on sterile paper and incubated at 34°C in sterile net-covered cages, 200-400 eggs per cage. The eggs hatch after 4 to 6 days and the larvae are then ready for use.

When used to isolate rickettsiae or other agents of disease from a patient, such a cage of larvae is fed sterilely and twice daily over a period of four to twelve days, depending on the disease being investigated. To do this, the net side of the cage is attached to the sterilized skin on the medial side of the forearm or shank. Sterilization is affected with ether followed by 70% alcohol, care being taken to be sure that the alcohol has completely evaporated before feeding begins. Bacterial sterility is routinely checked by seeding larval excrement to agar plates.

At the conclusion of the 4- to 12-day feeding period, the cage is opened and note made of the living and dead larvae, especial attention being given those that are red or red-black in color. Infectivity is sought in the accumulated excrements and in the sterilely resected intestines of the living and dead reddish larvae. Microscopic examination is carried out with the aid of contrast methods of Serkowski[1] (Nigrosin, 4% in H_2O) or by the Eisenberg[2] method.

In this way, we have isolated and observed the infectious bodies responsible for a number of virus and rickettsiae-like diseases. These bodies are, for the most part, round disks having about the size of typhus rickettsiae. Several of the diseases they produce are rheumatic in character (*Polyarthritis rheumatica acuta, Chorea minor*), though one gives a toxic diarrhea in infants. To identify what is seen in stained preparations with the diseases in question, we have made use of both agglutination and of the Bordet-Gengou reaction. Some isolations have produced symptoms in rabbits and sensitized guinea pigs. Inactivated suspensions have given as intracutaneous test prominent urticaria-like reactions in persons sensitized through chronic disease and have thus proved a useful means of diagnosis. In sensitive persons, this reaction also supplies an excellent test for the presence of traces of the agents in louse intestines. At all times, corresponding emulsions of healthy louse intestines have been used as controls.

An essentially similar procedure for isolating infectious material has used bed bug (*Clinicis lectularii*) instead of louse larvae. It often permits the isolation of agents that would be missed using louse larvae. In this case, small pieces of wood replace cloth as receivers of laid eggs and feeding is once a day for an hour. Each cage is set up for 60-80 eggs. Incubation takes place at a lower temperature (28-30°C).

This work was begun in the Typhus Institute of Professor R. Weigl and in the Children's Clinic of Professor Fr. Groer in Lwow. It has been continued in the Dermatologic Clinic of Professor Lenartowicz in Wroclaw.

1 Sterling-Okuniewski, *Technika badan bakteriologicznych* (Warzawa, 1922), p. 72.

2 Eisenberg, Ph., *Zentr. Bakt. I akt Originale Bd.*, 71, 421.

TABLE I

Resistance of Mice Immunized with Ultraviolet-Inactivated Psittacosis Virus.

Injections of vaccine	Challenge Injection				Result†	
	Volume	LD$_{50}$ Doses		Route	Vaccinated mice	Non-vaccinated controls
2	0.03 cc	4,000		Intracerebral	12/12	6/6
"	"	400		"	12/12	6/6
"	"	40		"	12/12	6/6
"	"	4		"	6/12	6/6
"	0.03 cc	40,000		Intraperitoneal	2/3	6/6
"	"	4,000		"	0/10	4/6
"	"	400		"	0/10	4/6
"	"	40		"	0/10	4/6
3	0.25 cc	48*		"	15/38	27/27
						26/26**

†Numerator *indicates number of deaths; denominator indicates number of mice inoculated.*
*LD$_{50}$ titer determined here by intraperitoneal inoculation; all others determined by intracerebral.
**This set of controls received normal allantoic fluid at times of immunization.

TABLE II

Resistance of Mice Immunized with Ultraviolet-Inactivated Human Pneumonitis and Ornithosis Viruses.

Virus	LD$_{50}$ Doses of Challenge Injection (0.03 cc, intracerebral)	2 inj. of vaccine		3 inj. of vaccine	
		Vaccinated	Control	Vaccinated	Control
Human Pneumonitis	180	5/8	8/8	4/10	8/8
	18	0/7	8/8	0/10	8/8
Ornithosis	900			8/10	6/6
	90	3/9	6/6	1/10	6/6

virus was given. Resistance to intracerebral challenge was definitely demonstrated in mice which had received human pneumonitis and ornithosis vaccines (Table II).

Conclusions. These studies demonstrate that it is possible to inactivate completely certain viruses of the psittacosis group in less than one second by exposure to appropriate intensities of ultraviolet irradiation. Such preparations retain antigenicity as indicated by their ability to immunize mice against challenge doses of active virus.

16029 P

Techniques for Rickettsial and Virus Cultivation.

JOZEF KUBICZ. (Introduced by R. W. G. Wyckoff.)

Wroclaw, Poland.

The following note describes modifications made in the Weigl method for cultivating rickettsiae and other small agents of disease within susceptible lice. Made primarily to facilitate isolations from diseased persons, they involve feeding larvae reared under sterile conditions on potentially infected blood.

The technique is essentially as follows. From 200-400 adult lice (*Pediculus vestimenti*) are washed for 2 minutes in 60% alcohol and subsequently with sterile physiological saline. They are then enclosed in a cage (6 x 3 x 1 cm) covered on one side with a fine net and containing within it the piece of

cloth recommended by Weigl. The caged lice are fed twice daily on a healthy nourisher whose skin is first sterilized with 70% alcohol. Between feedings the cages are kept in sterile paper boxes at 34°C. Under these circumstances, masses of eggs are laid on the cloth. These eggs are removed and washed first with 30% alcohol or other suitable weak disinfectant and then with sterile saline, bacterial sterility of the washed eggs being checked by culturing the last drops of the saline wash on nutrient agar. The sterile eggs are dried on sterile paper and incubated at 34°C in sterile net-covered cages, 200-400 eggs per cage. The eggs hatch after 4 to 6 days and the larvae are then ready for use.

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1 Sterling-Okuniewski, *Technika badan bakteriologicznych* (Warzawa, 1922), p. 72.

2 Eisenberg. Ph., *Zentr. Bakt. I akt Originale Bd.*, 71, 421.

16030

Blood, Plasma, and "Drawn Blood" Volumes in the Rat.

Richard W. Lippman.* (Introduced by T. Addis.)

From the Department of Medicine, Stanford University School of Medicine, San Francisco, Calif.

The following simple method for determination of circulating blood and plasma volumes has been used in the normal albino rat. It can be applied usefully to control and experimental rats just before they are killed.

The rat was anesthetized with ether in a Mason jar. As soon as the animal ceased to move, the inferior vena cava was exposed rapidly by cutting the abdominal cavity widely open with scissors. Light anesthesia was continued by applying a small beaker containing ether-soaked cotton over the nose. A hemoglobin solution of known volume and concentration was injected slowly into the vena cava from a tuberculin syringe.† The volume was varied from 0.20 to 0.50 cc, depending upon the size of the rat, and the concentration varied from 7% to 8%. The needle was kept in place to prevent leakage for two minutes. It was then withdrawn and the animal was exsanguinated by severing the abdominal aorta. The first ml of blood was collected in a tube containing 2 mg of potassium oxalate, for a hematocrit determination by the Wintrobe method. The remaining blood was collected carefully, until all bleeding stopped, in an oiled centrifuge tube and allowed to clot. Under these conditions we had previously found that hemolysis did not occur.

The total "drawn blood' was recorded. The clotted blood was centrifuged and the serum separated for measurement of its oxyhemoglobin concentration by the method of Evelyn and Malloy.[1] From these data plasma and blood volumes were calculated and corrected by subtracting the volume of hemoglobin solution injected.

The procedure outlined was selected, in spite of known objections, because it happened to fit our experimental needs, and required simple analytical techniques with which we were fully familiar. Essentially, it is a dye method in which blood is drawn for measurements of concentration and hematocrit after a suitable mixing time. Preliminary experiments showed that a 2-minute mixing time gave the same results as longer mixing times. Since the interval was short and the dye was one that is essentially nondiffusible and relatively slowly metabolized, use of a mixing curve, such as that of Noble and Gregerson[2] seemed unnecessary and technically would be almost impossible to obtain.

It was also found that skill of the operator was of some importance in obtaining reproducible results. At first determinations will tend to be low, presumably due to shock resulting from slow and inexpert handling of the animals. However, with a reasonable amount of practice, relatively constant and repeatable results are obtained.

Results. Blood volumes (BV) and plasma volumes (PV) were determined in 125 rats (47 female, 78 male) ranging from 48 to 308 g. In 111 the "drawn blood' volume was recorded. As there did not appear to be any significant difference between the sexes, the results were pooled. Plots of PV and BV against body weight in grams (BW) and body surface in square centimeters (BS) do not produce a straight line, but when log BV and log PV are plotted against log BW (Fig. 1) the points fit a straight line reasonably well. From the data obtained, regression lines were

1 Evelyn, K. A., and Malloy, H. T., *J. Biol. Chem.*, 1938, 126, 655.

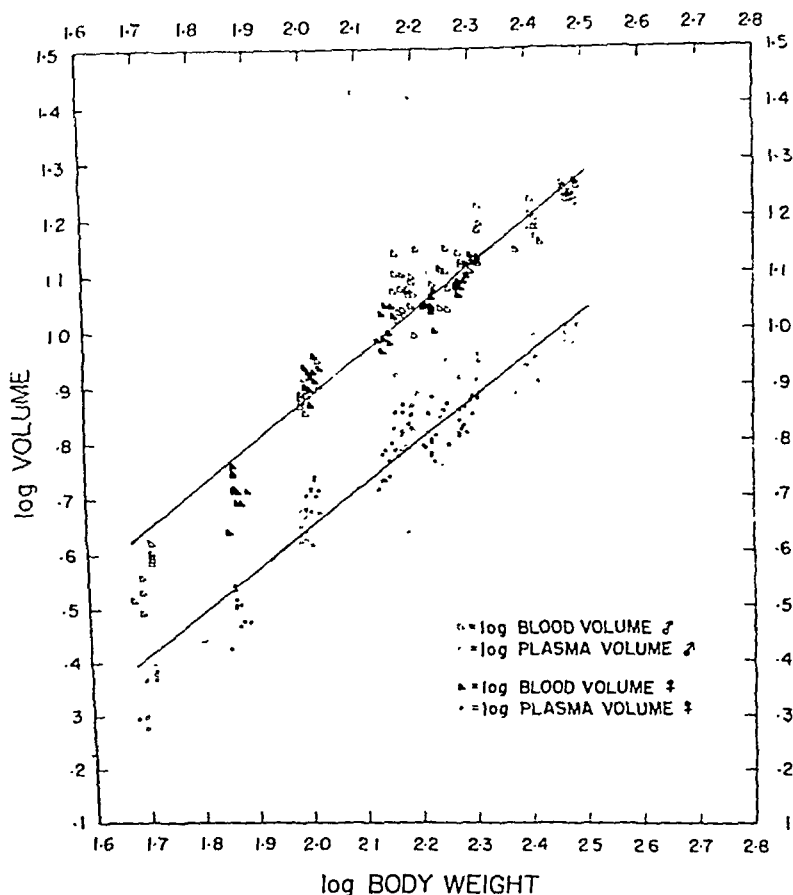2 Noble, R. P., and Gregerson, M. I., *J. Clin. Invest.*, 1946, 25, 158.

FIG. 1.

calculated, and the following formulae were established as applicable to the observed weight range:

BV = 0.195 BW $^{0.704}$
PV = 0.122 BW $^{0.778}$

Nearly all of the observations fell within a reasonably small zone on either side of the line, the values falling below the line at weights below 100 g. Although the relation of blood and plasma volume to a power of body weight was not perfect, it was a better fit than any simple alternative, and is presented for purposes of comparison with other investigators' results.

The general relationship of organ size to a power of body size has been established by Huxley,[3] who used body size from which the

size of the organ has been subtracted. The use of total body weight is of considerably greater convenience and in the present instance, where the weight of the blood is a relatively small proportion of the total body weight, the difference introduced by such use was found to be insignificant. Failure of the regression line to fit more perfectly may be due to increased deposition of fat, a relatively avascular tissue, as the rats approach maturity and senescence.

Comparison of our results with those published previously is complicated by the various methods of presenting data. Table I summarizes in chronological order the results of several different observers and, for purposes of comparison, gives the blood and plasma volumes calculated by us for a rat of 150 g. In the references cited, body surface

[3] Huxley, J. S., *Problems of Relative Growth*, New York. The Dial Press, 1932.

16030

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1 Evelyn, K. A., and Malloy, H. T., *J. Biol. Chem.*, 1938, **126**, 655.

2 Noble, R. P., and Gregerson, M. I., *J. Clin. Invest.*, 1946, **25**, 158.

volume at weights below 100 g and somewhat above 50% at high weights. This confirms the impression acquired incidentally in this laboratory, in the process of accumulating data in numerous other rat experiments. that an indication of the direction of change in actual blood volume can be obtained by measuring the "drawn blood" volume.

Summary. 1. Blood and plasma volumes have been determined in the normal albino rat, and have been found to be approximately related to a power of body weight, the deviation being ascribed to deposition of avascular fat as the rat matures. There appeared to be no significant difference in this regard between the sexes.

2. An estimation of blood volume changes may be made from the "drawn blood" volumes.

16031

Effect of Chloroform on the Antitryptic Activity of Blood Plasma.

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It was shown in a previous paper[1] that treatment of plasma with 10% chloroform, decreased its hypertensinase activity instead of increasing it. This was a remarkable finding because: (a) hypertensin is a very sensitive reagent to test for proteolytic activity, and (b) the characteristic clotting and fibrinolytic properties of chloroform treated plasma have been attributed to an increased tryptic activity.

Previous experiments have also shown that hypertensin is an excellent substrate for the detection of antiproteolytic substances. Using hypertensin as a reagent, we have been able to show that plasma protects hypertensin against the destructive action of trypsin and to a lesser degree from that of chymotrypsin. On the contrary, the hypertensinase activity of crystalline carboxypeptidase and pure aminopeptidase (from yeast) remained unaffected (Croxatto and Croxatto[2]).

The degree of protection afforded by plasma, against the destruction of hypertensin by an appropriate dose of trypsin, may be used as a measure of its antitryptic activity. By using a technique based on this assumption, we have studied the effect of chloroform treatment on the antitryptic activity of plasma.

Experimental. Dog, ox, horse and human plasmas were treated with 10% chloroform, with the technique already described. Chloroform was separated by centrifuging and vacuum distillation, usually after 24 hours contact with the plasma. Aliquots of treated and untreated plasma were added to hypertensin (3 units) plus 0.1 ml of a solution of crystalline trypsin. A freshly prepared solution of one mg of trypsin* per milliliter of 0.9% sodium chloride was used. The amount of this solution used (0.1 ml) was enough to produce more than 80% destruction of hypertensin in 2 hours. with no plasma present.

The action of plasma on hypertensin, in absence of trypsin. was also tested. All experiments were carried out at pH 7.4. obtained by adding 0.2 ml of a solution of 0.02N sodium phosphate buffer, pH 7.4. The incubation period was of 2 hours at a temperature of 37°C.

The hypertensin activity present after incubation was determined by injecting the solutions into the femoral vein of a cat. under Dial anesthesia and recording with the

[1] Sainz, N., and Croxatto, H., *Bol. Soc. Biol.,* Santiago, Chile, 1944, **2**, 261.

[2] Croxatto, H., and Croxatto, R., *Bol. Soc. Biol.,* Santiago, Chile, in press.

* Plant's crystalline trypsin containing 85% of magnesium sulfate.

TABLE I.

| Author | Formulæ | | 150 g rat | |
	BV	PV	BV cc	PV cc
Chisolm[5]	.099 BW$^{0.9}$		9.0	
Went and Drinker[6]	.074 BW		11.1	
Cutting and Cutter[7]		.014 BS		4.5
Griffith and Campbell[8]	.043 BW		6.5	
Beckwith and Chanutin[9]	.054 BS	.027 BS	17.2	8.7
Metcoff, Favour and Stare[10]	.047 BS	.028 BS	15.2	8.7
Lippman	.195 BW$^{0.794}$	.122 BW$^{0.778}$	10.4	6.0

BV is blood volume in cc, PV is plasma volume in cc, BW is body weight in grams, BS is body surface in sq.cm.

was assumed to have been calculated by the formula of Carman and Mitchell[4] where not otherwise stated.

The "drawn blood" volumes obtained by measurement have been plotted in Fig. 2 against the blood volumes determined by the hemoglobin method described. The curve obtained indicates that the "drawn blood" volume is related to actual blood volume, being somewhat below 50% of the actual blood

Relation of "Drawn Blood" Volume to
- ACTUAL Blood Volume in Albino rats.

ACTUAL BLOOD VOLUME c.c.
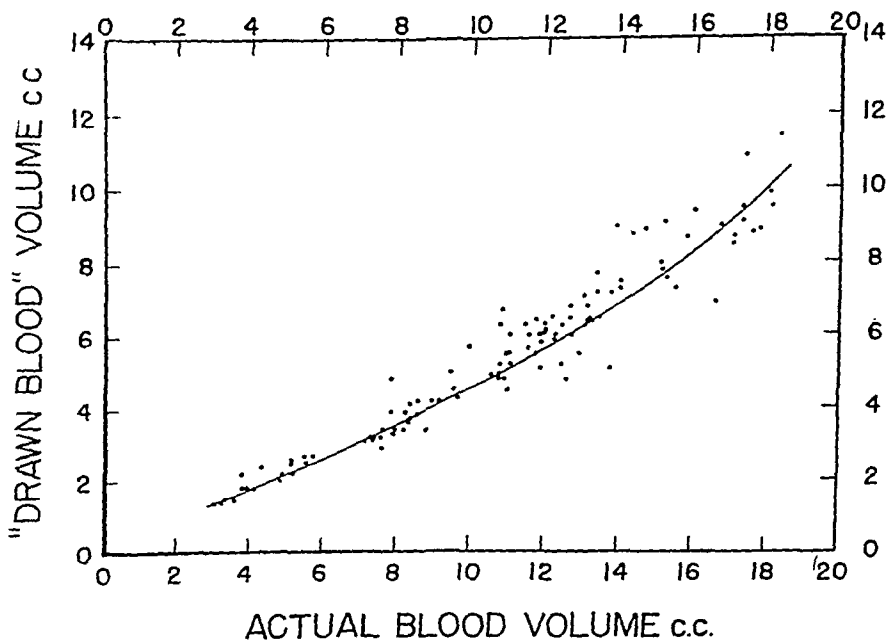
FIG. 2.

4 Carman, G. G., and Mitchell, H. H., Am. J. Physiol., 1926, 76, 380.

5 Chisolm, R. A., Quart. J. Exp. Physiol., 1911, 4, 207.

6 Went, S., and Drinker, C. K., Am. J. Physiol., 1929, 88, 468.

7 Cutting, W. C., and Cutter, R. D., PROC. SOC. EXP. BIOL. AND MED., 1935, 32, 1053.

8 Griffith, J. P., Jr., and Campbell, R., PROC. SOC. EXP. BIOL. AND MED., 1937, 36, 38.

9 Beckwith, J. R., and Chanutin, A., PROC. SOC. EXP. BIOL. AND MED., 1941, 46, 66.

10 Metcoff, J., Favour, C. B., and Stare, F. J., J. Clin. Invest., 1945, 24, 82.

16032

The Serum Uric Acid Concentration in Essential Hypertension.*

JOSEPH R. STANTON AND EDWARD D. FREIS.   (Introduced by Robert W. Wilkins.)

From the Evans Memorial, the Surgical and Medical Services, Massachusetts Memorial Hospitals, and the Departments of Surgery and Medicine, Boston University School of Medicine.

The blood uric acid concentration in patients with arterial hypertension has not received careful attention since early investigators reported the uric acid level to be increased.[1-4] This reported increase was ascribed to metabolic factors and not to beginning renal insufficiency.[3] More recent work on methods for the determination of the serum concentration of uric acid indicated that values determined by earlier methods contained many sources of potential error.[5-8] Further, many of the subjects previously studied had varying degrees of cardiac and renal failure.

If there is a metabolic fault in essential hypertension that results in hyperuricemia it would be of importance as a lead to further investigation. However, it was considered necessary first to reevaluate the question using modern methods of analysis and paying particular heed to the presence of renal insufficiency.

Material and Method. Fifty consecutive hypertensive patients admitted to the Ward and Private services of the Massachusetts Memorial Hospitals for lumbo-dorsal splanchnicectomy were the subjects of this study (c. f. Table I). Chronic glomerulonephritis and other forms of primary renal disease were

TABLE I.

	No. of cases
Males	20
Females	30
Age group	
20-30	4
30-40	13
40-50	19
50-60	13
60-70	1
Systolic blood pressure over 180 mm Hg.	42
Diastolic blood pressure over 100 mm Hg.	48
Proteinuria	20
Nitrogen retention	2

adequately ruled out by history and laboratory examinations.

Hypertension had been known to exist in these patients for from 6 months to 20 years. The highest blood pressure recorded on admission was 250/160, the lowest 160/96. Only one patient was in cardiac failure during the period of observation.

The determination of uric acid was carried out by the method of Folin,[7] adapted to the Klett Summerson photoelectric colorimeter† using a protein free filtrate of serum.[9] Blood samples from all patients in this series were taken in the post-absorptive state. Normal values obtained by this method ranged from 2-5 mg/100 cc of serum.

Results. Persistent hyperuricemia was demonstrated in only 2 cases or 4% of the series. Normal serum uric acid levels were found on the initial determination in 44 cases. Six cases exhibited a serum uric acid level above 5 mg % at the time of the initial determination. On repeat examination carried out 24 to 48 hours later, 4 of these 6 cases were found to have uric acid levels well within the normal

1 Kylin, E., *Acta med. Scandinav.*, 1923, 58, 342.

2 Hitzenberger, K., Richter and Quinter, M., *Wien. Arch. f. inn. Med.*, 1921, 2, 189.

3 Fishberg, A. M., *Arch. Int. Med.*, 1924, 34, 503.

4 Williams, J. L., *Arch. Int. Med.*, 1921, 27, 748.

5 Wu, H., *J. Biol. Chem.*, 1922, 51, 21.

6 Folin, O., *J. Biol. Chem.*, 1934, 106, 311.

7 Folin, O., *J. Biol. Chem.*, 1933, 101, 111.

8 Dana, E. S., Bosnes, R. W., and Dill, L. V., *J. Clin. Invest.*, 1944, 23, 776.

9 Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, 38, 81.

usual technique the changes in blood pressure in the carotid artery. All solutions were boiled and then centrifuged, using supernatant. This treatment eliminates completely the secondary effects of foreign proteins and trypsin on the blood pressure. The sensitivity of the animal was checked, from time to time, with a standard solution of hypertensin.

The amount of plasma, not treated with chloroform, necessary to afford a 50% protection (1.5 units of hypertensin) varied between 0.04 and 0.06 ml. The hypertensinase activity of such a volume of plasma was negligible.

Results. As shown in Fig. 1, the treatment of plasma with chloroform decreases greatly its antitryptic activity. Although chloroform treatment does not eliminate completely the antitryptic activity, the decrease is larger after 24 than after one hour contact. The antitryptic activity of 0.5 ml of 24 hours treated plasma is approximately equivalent to that of 0.05 ml of untreated plasma.
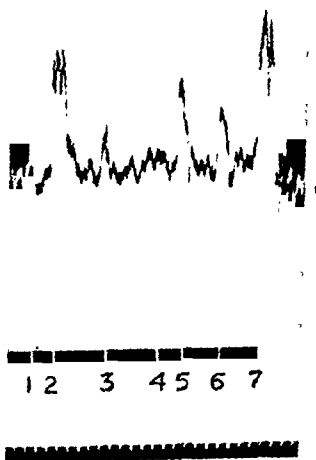

FIG. 1.

Discussion. Our results favor the hypothesis of an inactivation, by the chloroform treatment, of one or several substances acting as inhibitors of the pancreatic trypsin. Heparin cannot be considered as such an inhibitor because of not appreciably influencing the inactivation of hypertensin and pepsitensin by trypsin, whether plasma is present or not (Marsano, Croxatto, Croxatto[3]).

The clotting and proteolytic activities of chloroform treated plasma may be due to an inhibition of antitryptic substances. In fact, the addition of crystalline antitrypsin extracted from soy bean meal, counteracts the clotting and fibrinolytic action of chloroform treated plasma and certain snake venoms (H. Croxatto[4]).

The changes produced by the chloroform treatment are very complex and difficult to interpret. Several enzymatic activities of plasma are disturbed (Sainz and Croxatto[1]). and the interaction of hypertensinogen, renin, and hypertensinase is changed (Croxatto and Croxatto[2]). Furthermore, no explanation can be given of why hypertensin is destroyed more easily by non-treated plasma than by plasma treated with chloroform, while the proteolytic activity is increased by this treatment, as shown by the solubilization of the blood clot.

Summary. Treatment of human, dog, ox, and horse plasmas with 10% chloroform, decreases their antitryptic properties.† These properties were measured using hypertensin as substrate.

3 Marsano, A., Croxatto, R., and Croxatto, H., *Bol. Soc. Biol.*, Santiago, Chile, 1945, **2**, 350.

4 Croxatto, H., *Rev. Soc. argent. Biol.*, 1946, **22**, 477.

† While this work was in course of publication, a paper by L. R. Christensen appeared in *J. Gen. Physiol.*, 1946, **30**, 149, where he states that chloroform treatment of serum produces an immediate inactivation of "protease inhibitor."

16033 P

Kidney Damage in the Golden Hamster Following Chronic Administration of Diethylstilbestrol and Sesame Oil.*

V. S. Matthews, H. Kirkman, and R. L. Bacon.   (Introduced by C. H. Danforth.)

From the Department of Anatomy, Stanford University School of Medicine, Stanford, Calif.

Estrogen induced hyperplasias have been reported repeatedly for various species of animals. The most conspicuous of such changes reported so far for the estrogenized hamster are in the hypophysis. In this gland the pars intermedia develops very extensive adenomas which migrate through the stalk well into the hypothalamus (Vasquez-Lopez,[1] Koneff, Simpson and Evans,[2] Matthews[3]). For the hamster the only kidney alteration mentioned in the literature is found in the paper by Vasquez-Lopez where Table I records a "large secondary deposit macroscopically visible in the left kidney" of one of 28 hamsters implanted with 10 mg pellets of diethylstilbestrol or of estradiol benzoate; this particular animal had been treated for 299 days with the natural estrogen.

While a tumor-producing action, in guinea pigs, has been denied for sesame oil by Lipschütz and Vargas[4] it has been affirmed in man by Conrad, Conrad and Weiss.[5] To date we have had no opportunity of examining the kidneys of sesame oil-treated control hamsters. We know of no reports in the literature, however, which attribute the formation of renal adenomas, in any species, to treatment with either estrogens, sesame oil, or any other similar oil.

In this laboratory we have examined kid-

neys from 14 hamsters (*Cricetus auratus*) in which group were 4 untreated males, one untreated female, 6 treated males and 3 treated females. On the average the treated animals received a total of 41 mg of diethylstilbestrol in 25 cc of sesame oil followed by a total of 61 mg in the form of microcrystals in 23 cc of saline solution. Injections were made subcutaneously, daily or on alternate days, over a period averaging 379 days. The animals averaged 429 days in age and 112 g in body weight at the time of sacrifice.

Kidney changes were observed in all of the 9 treated animals. Of these changes by far the most conspicuous were adenomas (Fig. 1C) found in each of the 6 males. In size these tumors ranged from small nodules a few cells in width to masses about one-third of the volume of a control kidney. In many of the adenomas were groups of cells showing "colloid degeneration", rare in the cells of intact tubules. Many of the adenomas were in the region of the pelvis, projecting more or less into the renal sinus. The cell arrangement in some of these suggests an origin from the transitional epithelium of the pelvis. Other adenomas occurred in various portions of the kidney cortex. The fact that the adenomas alone of all the kidney tissue contain much sudanophilic substance suggests that sesame oil (also sudanophilic) may be the tumor-producing agent. Experiments are under way to test the relative actions of oil and estrogen and to investigate the cause of the sex difference in kidney response. In one animal the renal tumor had metastasized through the peritoneal cavity to the mesenteries and the spleen.

In 2 males, fibromas were observed in the capsular connective tissue.

No extraglomerular arteriosclerosis was observed, but in the females amyloid infiltration

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* The diethylstilbestrol used in this study was supplied through the courtesy of Dr. D. C. Hines of the Eli Lilly Company.

1 Vasquez-Lopez, E., *J. Path. and Bact.*, 1944, 56, 1.

2 Koneff, A., Simpson, M. E., and Evans, H. M., *Anat. Rec.*, 1946, 94, 169.

3 Matthews, V. S., 1947 unpublished data.

4 Lipschütz, A., and Vargas, L., *C. R. Soc. biol.*, 1939, 130, 9; Lipschütz, A., Rodriguez, F., and Vargas, L., *C. R. Soc. biol.*, 1939, 130, 939.

5 Conrad, A. H., Conrad, A. H., Jr., and Weiss, R. S., *J. A. M. A.*, 1943, 121, 237.
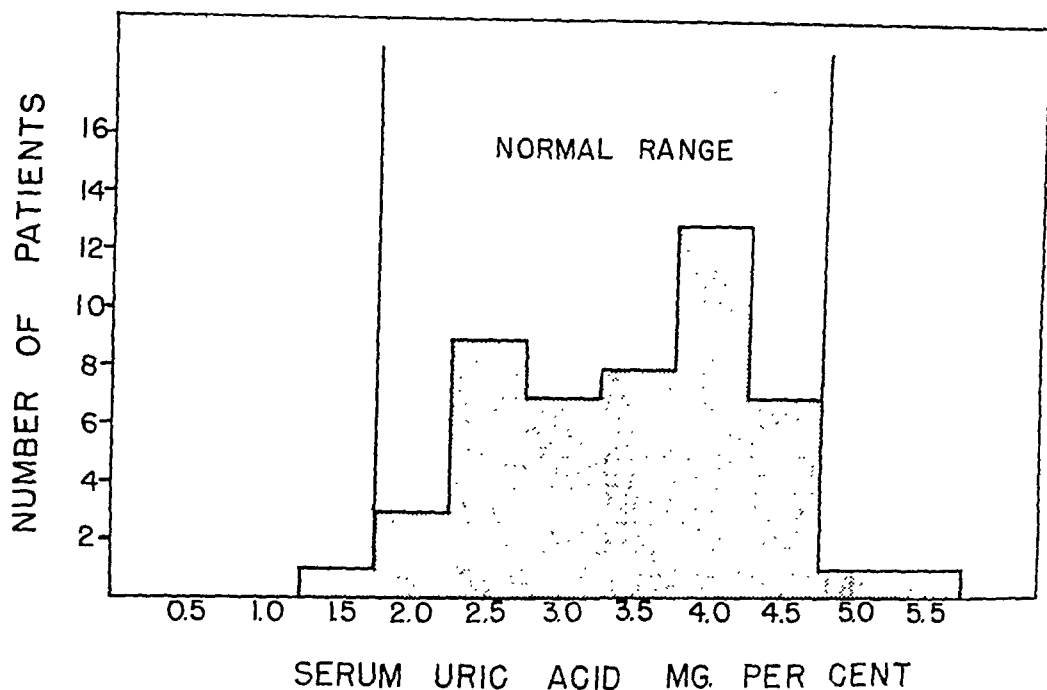
FIG. 1.
Serum uric acid concentration of 50 patients with essential hypertension.

range. The mean serum uric acid level for the entire series was 3.65 mg % with a standard deviation of 0.88 (Fig. 1).

Discussion. It is significant that the 2 cases with persistent hyperuricemia showed evidence of renal insufficiency as manifested by elevation of the blood non-protein-nitrogen. Proteinuria was present in both of these cases and also in 3 of the 4 cases showing transient hyperuricemia. However, hypertension and proteinuria were observed in 12 patients showing normal uric acid levels. Therefore, no consistent relationship was observed between proteinuria and elevated serum uric acid levels. The transient elevations of serum uric acid concentration observed in four cases were slight, none exceeding 5.5 mg %.

Summary and Conclusions. In a series of 50 cases essential hypertension, unless complicated by renal insufficiency, was associated with essentially normal serum uric acid values.

16034

Effect of Meningococcal Endotoxin on Histamine Content of Blood and Tissues of Rabbits.[*]

ERNEST KUN.   (Introduced by C. Phillip Miller.)

From the Department of Pharmacology and the Department of Medicine, University of Chicago.

The following studies were undertaken as part of an investigation of the biochemical changes which occur in the animal body after the intravenous injection of meningococcal endotoxin. The pathological condition produced in this way is known as toxemia and since different bacterial toxins seem to elicit changes in metabolism which have certain characteristics in common,[1] the results of these studies may be of some general interest.

The important role of histamine in the pathology of infectious diseases, as well as in conditions closely related to them, such as allergy and anaphylaxis, is well recognized; and has been reviewed by Best[2] and by Dragstedt.[3,4] Feldberg and Keogh[5] and Feldberg and Kellaway[6] reported experiments which showed that an increase in histamine liberation from isolated organs occurred when the perfusion fluid contained staphylococcal endotoxin. Kellaway, Trethewie and Turner[7] showed that the toxin of *Clostridium welchii* increased the histamine output of isolated organs of the cat and the rabbit. These results suggest that bacterial toxins interfere in some way with histamine metabolism.

The present study is concerned with the changes in the histamine content of the blood and tissues of rabbits injected intravenously with meningococcal endotoxin.

Materials and Methods. The meningococcal endotoxin, as prepared by Boor and Miller, is a mixture of nucleoproteins, globulins, albumins, and a highly toxic glycolipid.[8,9,10] Endotoxin was prepared from type I meningococcus grown for 18 to 20 hours on casein-digest agar. The microorganisms were washed thrice in saline, resuspended in water, brought to pH 8.2 to 8.5 and kept at a temperature of about 27°C for 2 or 3 hours and in the refrigerator overnight. The suspension was then neutralized and sterilized by heating 2 or 3 times at 60° C for 25 minutes. Sterility was always proved by culture. This toxin mixture was heat stable,[11] and could be kept in the icebox for several months without much change in toxicity. Since the chemical nature of meningococcal endotoxin is not yet known, it seemed best to use the original protein-glycolipid suspension. The rabbits were injected intravenously with a dose sufficient to cause death within a few hours and this dose is recorded in ml per kg body weight. The solid content of the toxin was 1.14% as determined by Boor. These experimental conditions can be considered as acute meningococcal endotoxin poisoning.

1 Holmes, E., *Physiol. Rev.*, 1939, **19**, 439.

2 Best, C. H., *Physiol. Rev.*, 1931, **11**, 371.

3 Dragstedt, C. A., *The Significance of Histamine in Anaphylaxis and Allergy.* 1943.

4 Dragstedt, C. A., *The Role of Histamine in Various Pathological Conditions and the Methods Controlling Its Effects.* 1945.

5 Feldberg, W., and Keogh, V., *J. Physiol.*, 1937, **90**, 280.

6 Feldberg, W., and Kellaway, C. H., *Australian J. Exp. Biol. Med. Sci.*, 1938, **16**, 219.

7 Kellaway, C. H., Trethewie, E. R., and Turner, W., *Australian J. Exp. Biol. Med. Sci.*, 1940, **18**, 253.

8 Boor, A. K., and Miller, C. P., *J. Exp. Med.*, 1934, **59**, 63.

9 Boor, A. K., and Miller, C. P., *Arch. Path.*, 1941, **29**, 724.

10 Boor, A. K., and Miller, C. P., *J. Inf. Diseases*, 1944, **75**, 47.

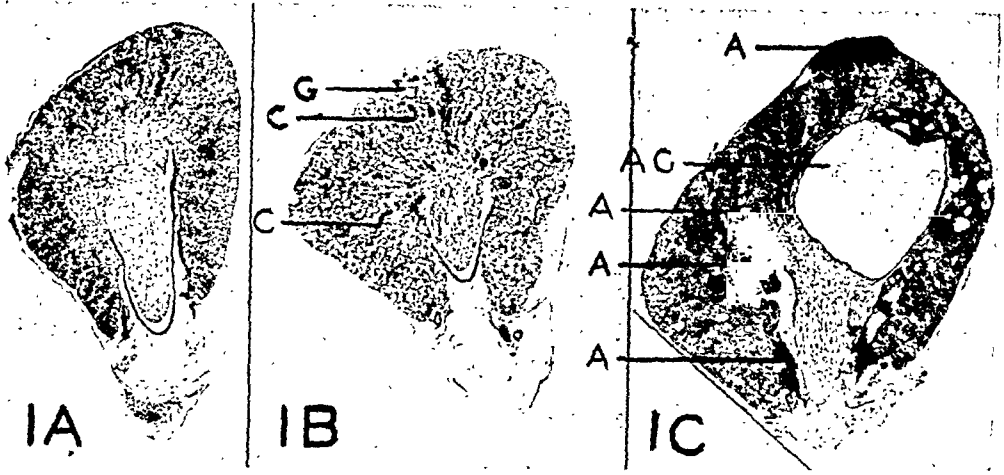11 Miller, C. P., Becker, R. M., Schad, Doretta, and Robbins, M. Wright, *J. Inf. Dis.* 1943, **73**, 248.

FIG. 1.

Fig. 1, A to C, (all ×5) are transverse sections through kidneys of golden hamsters. 1A represents a normal kidney from a 389-day-old ♀ weighing 136 g; untreated. 1B is from a 364-day-old ♀ weighing 85 g; received 29 mg of diethylstilbestrol in 17 cc of sesame oil over a period of 107 days, followed by 59 mg in the form of microcrystals suspended in 20 cc of saline solution over a period of 215 days. 1C is from a 456-day-old ♂ weighing 130 g; received 55 mg of diethylstilbestrol in 33 cc of sesame oil over a period of 205 days, followed by 58 mg of microcrystals in 19 cc of saline solution over a period of 215 days. A, adenoma; Ac, cyst in adenoma; C, casts in enlarged tubules; G, enlarged capsular space about glomerulus.

was considerable in many glomeruli and around many convoluted tubules. This was associated with large and abundant hyaline casts and tubular atrophy (Fig. 1B). In 2 of the 6 treated males also, amyloid was demonstrated. In one animal it was confined to traces in a few of the glomeruli; in the other animal, No. 49, (the one possessing fewest and smallest adenomas) it was present in somewhat greater amount and found around convoluted tubules as well as in glomeruli. A similar picture of glomerulo-nephritis has been described by Korenchevsky and Ross[6]

6 Korenchevsky, V., and Ross, M. A., Brit. Med. J., 1940, 1, 645.

in both male and female, normal and gonadectomized, albino rats treated with 0.018 to 0.2 mg of estradiol dipropionate, in sesame oil, weekly for from 21 days to 3¾ months. They make no mention of sesame oil controls or of sexual differences in kidney reaction.

A more detailed account, involving hamsters still being treated, will be published later.

Summary. The kidneys of golden hamsters treated for long periods with diethylstilbestrol and sesame oil tend to undergo marked changes of a destructive character. In the male these changes are primarily in the direction of potentially malignant tumor formation. In the female they are in the direction of glomerulo-nephritis

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Effect of Meningococcal Endotoxin on Histamine Content of Blood and Tissues of Rabbits.*

Ernest Kun. (Introduced by C. Phillip Miller.)

From the Department of Pharmacology and the Department of Medicine, University of Chicago.

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4 Dragstedt, C. A., *The Role of Histamine in Various Pathological Conditions and the Methods Controlling Its Effects*, 1945.

5 Feldberg, W., and Keogh, V., *J. Physiol.*, 1937, 90, 280.

6 Feldberg, W., and Kellaway, C. H., *Australian J. Exp. Biol. Med. Sci.*, 1938, 16, 219.

7 Kellaway, C. H., Trethewie, E. R., and Turner, W., *Australian J. Exp. Biol. Med. Sci.*, 1940, 18, 253.

8 Boor, A. K., and Miller, C. P., *J. Exp. Med.*, 1934, 59, 63.

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10 Boor, A. K., and Miller, C. P., *J. Inf. Diseases*, 1944, 75, 47.

11 Miller, C. P., Becker, R. M., Schad, Doretta, and Robbins, M. Wright, *J. Inf. Dis.*, 1943, 73, 248.

EFFECT OF MENINGOCOCCAL ENDOTOXIN ON THE BLOOD HISTAMINE .OF RABBITS
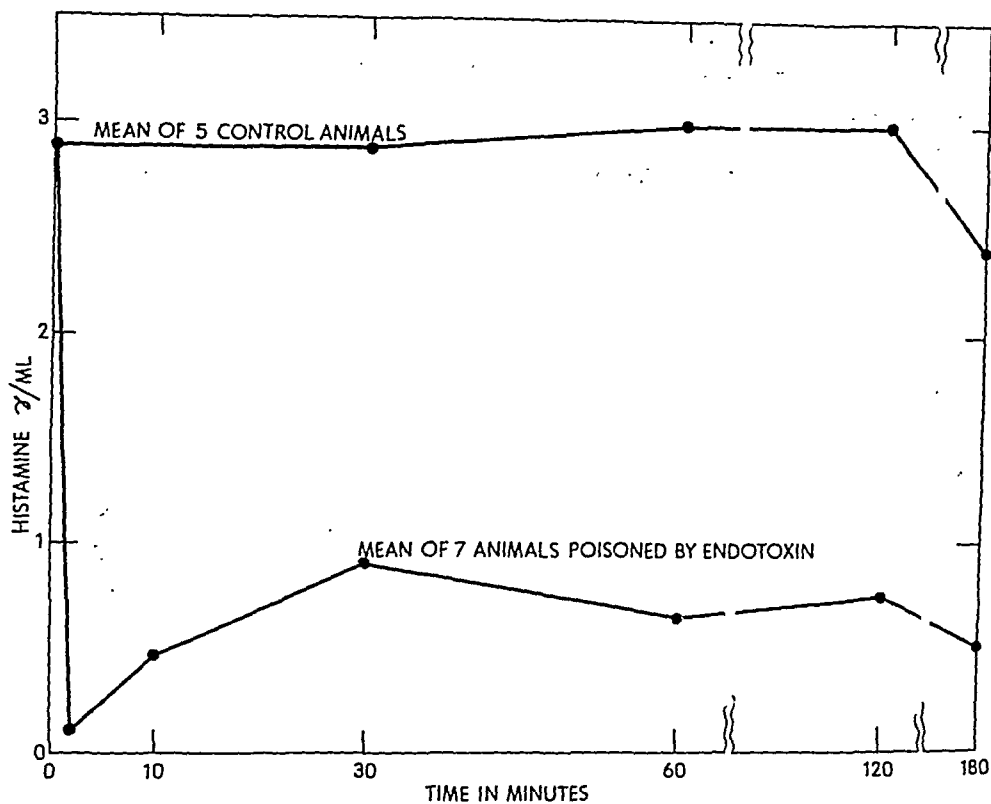
FIG. 1.

Thirty-eight rabbits, weighing from 2.4 to 3.7 kg were used as experimental animals. No anesthesia was used, because it was found that narcosis itself caused significant changes in the blood histamine content of the rabbit.† Blood was removed by cardiac puncture and immediately delivered into a weighed amount of 10% trichloracetic acid. The extraction was made shortly afterwards. Blood histamine was determined according to the method of Barsoum and Gaddum,[12] using the modification suggested by Code.[13] The histamine content of the tissues was determined on weighed samples ground in a glass tissue homogenizer[14]

in 10% HCl. Lipoids were removed from the tissue homogenates by ether extraction. The isolated ileum of the guinea pig, suspended in atropinized Tyrode at 37°C, was used as test object and the height of contraction was read directly on a scale. Each figure given in the tables and graphs is the mean of 5 parallel measurements (the S.D. did not exceed ±15%). In all measurements, the effect of known amounts of histamine phosphate was compared with the tissue extracts, and the results were expressed in terms of histamine base.

Results. Control Observations. In 5 normal rabbits the histamine content of the blood was determined at 0, 30, 60, 120, and 180 minutes. There was no significant variation in the blood histamine level during the course of

† Unpublished experiments.

12 Barsoum, G. S., and Gaddum, G. H., J. Physiol., 1938, 85, 1.

13 Code, C. F., J. Physiol., 1937, 80, 257.

BLOOD HISTAMINE OF RABBITS AFTER INTRAVENOUS INJECTION OF HISTAMINE
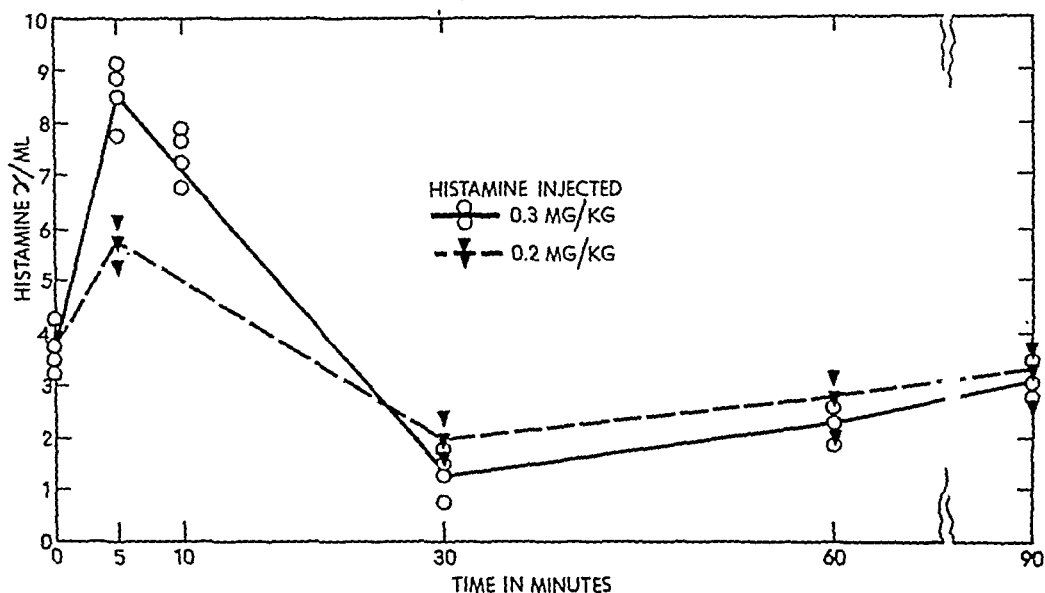
FIG. 2.

the experiment.

In the first series of experiments the concentration of histamine was studied in the blood of 7 rabbits, following the intravenous injection of 3.6 ml of endotoxin per kg body weight.

In all experiments the histamine content of the blood fell sharply immediately after the intravenous injection of endotoxin and remained well below its normal level as long as the animal lived—a period of 2-3 hours. (Fig. 1)

The mechanism involved in the disappearance of the blood histamine seems to be rapid. Since the endotoxin was the only foreign substance introduced into the animal body in these experiments, it might be supposed that endotoxin inactivates histamine. It was found, however, that when various amounts of histamine phosphate were added to the toxin, they could be recovered without significant loss by the same extraction method used for the blood analyses.

In the second series of experiments, the effects of simultaneous injections of histamine and endotoxin were studied in 7 rabbits. In

each experiment a control observation was first made by injecting histamine alone. When 0.3 mg histamine per kg body weight, in the form of histamine phosphate, was injected intravenously, the blood histamine content rose in 5 minutes to a level 2 to 3 times the normal. A 50% rise in blood histamine level was caused by an injection of 0.2 mg histamine per kg. (Fig. 2)

When meningococcal endotoxin (3.6 or 1.8 ml per kg) was injected simultaneously with a dose of histamine, which varied from 0.3 to 0.1 mg per kg no increase in blood histamine occurred. In each instance it fell to a significant degree. (Fig. 3)

The simultaneous injection of meningococcal endotoxin plus histamine markedly shortened the survival time of the rabbits. A dose of endotoxin, which usually caused death in 7-10 hours (1.8 ml toxin per kg), when injected simultaneously with 0.3-0.15 mg histamine per kg killed the animal in 5-10 minutes. These amounts of histamine alone injected intravenously were never fatal.

In the third series of experiments, determinations were made of the histamine con-

BLOOD HISTAMINE AFTER SIMULTANEOUS INJECTION OF HISTAMINE + MENINGOCOCCAL ENDOTOXIN
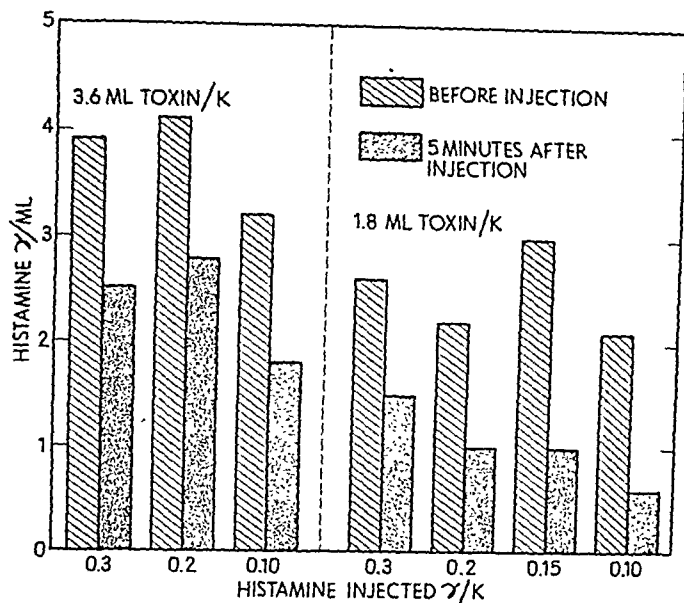
FIG. 3.

tent of the tissues of rabbits poisoned by meningococcal endotoxin. Seven animals were injected intravenously with 3.6 ml meningococcal endotoxin per kg, and their tissues analyzed immediately after death. The results are given in Fig. 4, which includes, for comparison, the histamine content of the organs of 5 normal rabbits. A significant increase in muscle and liver histamine was observed. The histamine content of the liver, as well as that of the diaphragm, showed about a 4-fold increase over the normal, while the skeletal muscle showed an average increase of 2.4 times the normal.

Discussion. These experiments show that the intravenous injection of meningococcal endotoxin into rabbits is followed by a rapid decrease in the histamine content of the blood and a considerable increase in the histamine content of the liver and muscle. The decrease in histamine content of the blood amounted to 2.75 histamine per ml of blood. If one assumes the total blood volume of a 3 kg rabbit to be 7% of its body weight[14] the total quantity of histamine which disappeared from

the blood was 0.77 mg; *i.e.*, 0.26 mg per kg. Based on the results of Levin and co-workers[15] who determined the average weight of rabbit organs, the amount of histamine accumulated in the tissues was about 5 mg. (1.7 mg histamine per kg in a 3 kg rabbit.) Since the amount of histamine which disappeared from the blood is about 1/7 the amount found in the tissues of poisoned animals, it is probable that most of the increased tissue histamine did not originate from the blood but was formed in the tissues themselves. The tissues of the poisoned rabbits contained about 3 times more histamine than the lethal intravenous dose of this substance[16] which circumstance suggests that histamine may be responsible for the death of the animals. These experiments, however, do not provide con-

14 Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, 1936, **116**, 495.

15 Levine, C. J., Mann, W., Hodge, H. C., Ariel, I., and DuPont, O., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 31S.

16 Sollmann, T., and Hanzlik, P. J., *Introd. to Exp. Pharmacol.*, 1928, p. 284.

HISTAMINE CONTENT OF TISSUES OF RABBITS POISONED BY ENDOTOXIN
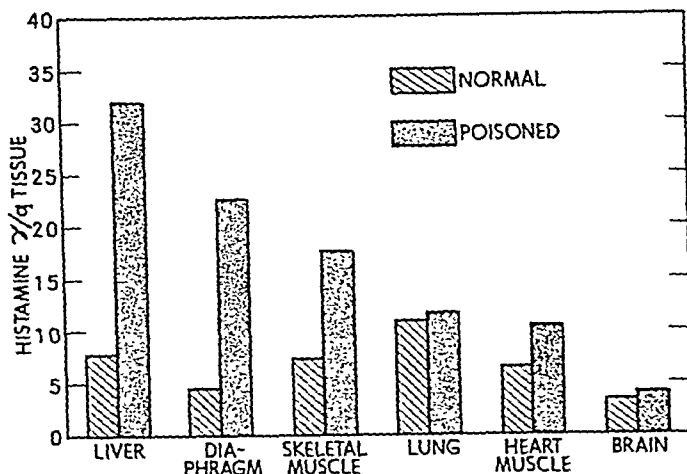
FIG. 4.

clusive proof of this supposition.

Summary. Meningococcal endotoxin injected intravenously into rabbits caused a rapid decrease in the histamine content of the blood and an increase in the histamine content of liver and muscle.

The decrease in histamine in the blood was not prevented by the intravenous injection of histamine along with the endotoxin.

The survival time of rabbits injected intravenously with meningococcal endotoxin was markedly shortened by a simultaneous injection of a small dose of histamine.

The author wishes to take this opportunity to thank Dr. C. Phillip Miller and Dr. Alden K. Boor of the Department of Medicine for supplying the meningococcal endotoxin and for their advice regarding these experiments.

16035

Effect of Oral Streptomycin on the Intestinal Flora.

L. W. KANE AND G. E. FOLEY,   (Introduced by L. Dienes.)

From the Departments of Medicine and Pathology and Bacteriology, Massachusetts General Hospital, and the Department of Bacteriology and Immunology, Harvard Medical School, Boston, Mass.

The majority of evidence seems to indicate that *E. coli* plays a predominant role in the pathogenesis of peritonitis following large bowel surgery.[1] Among the other microorganisms most frequently encountered are the fecal streptococci (Lancefield Group D streptococci), and *Clostridium welchii.*[1] With these facts in mind, it seems logical to suppose that the incidence of post-operative peritonitis would be reduced if the *E. coli* present in the bowel were eliminated. The less readily absorbable sulfonamides such as sulfaguanadine, sulfasuxidine and sulfathaladine have been used in an endeavour to accomplish this.

1 Meleney, F. L., Harney, H. D., and Jern, H. Q., *Arch. Surg.*, 1931, 22, 1.

Streptomycin as an antibacterial agent in the intestine, has the following properties: (1) soluble in water, (2) non-absorbable through the intestine, (3) non-toxic, (4) active in the presence of intestinal contents. Six cases were selected for study. Three of these had ulcerative colitis and 2 were pre-operative cases—a uretero-enterostomy and a large bowel resection. One was a normal individual. The 2 cases of ulcerative colitis, one pre-operative large bowel resection, and one normal individual were each administered 1.0 g of streptomycin *per os* daily, in 2 divided doses (0.5 g dissolved in a half-glass of water, morning and evening). The pre-operative uretero-enterostomy received 0.4 g of streptomycin *per os* 5 times daily. The third case of ulcerative colitis received streptomycin as a lavage per rectum; 20 ml containing 0.005 g streptomycin per ml was administered twice daily in an attempt to rid the distal segment of an old colostomy of *E. coli*.

Aerobic and anaerobic cultures were planted daily with a generous amount (approximately 0.5 g) of fresh stool collected from each patient. Bacterial counts per gram of wet stool were estimated from duplicate horse blood agar plates streaked with 0.05 ml of a 1:1,000,000 dilution of stool. In the 2 pre-operative cases who received streptomycin, swabs taken directly from the mucosa of the colon at operation were planted in aerobic and anaerobic cultures.

The results obtained in all 6 cases were essentially similar. A representative case is summarized in Table I. As can be seen in the Table, *E. coli* disappeared from the stool after streptomycin had been administered for 2 days. In one case, a normal individual, *E. coli* disappeared in one day, and in the remaining 4 cases, these microorganisms could not be cultured after 2 days on streptomycin.

Since microscopic examination of the stool showed an abundance of Gram negative rods even though *E. coli* could not be cultivated in aerobic or anaerobic media, daily motility tests were done on freshly collected stool specimens in order to ascertain whether or not these microorganisms actually were dead. In

TABLE I.
Effect of Oral Streptomycin on the Intestinal Flora.

Day of study	G streptomycin	Microscopic		Cultural—Aerobic and anaerobic					Aerobic colony count*
		Bacteria	Motility	E. coli	Streptococci	Bacteroides	Clostridia	Candida	
0	0	+++++++	++	++				0	—
1	1.0	+++++++	++	+++				0	52.4†
2	1.0	+++++++	±‡0	0	+++++++	+++++++	+++++++	0	6.6‡
3	1.0	+++++++	0	0				+	0
4	0.5	+++++++	0	±				+	
5	0		+	+				+	
6	0		++	++				+	

* Per g of wet stool in billions.
† *E. coli* predominant.
‡ Streptococci predominant.

all cases, when *E. coli* could no longer be cultivated from the stool, wet smears made directly from the specimen failed to show motile microorganisms. The failure of *E. coli* to grow in the presence of sodium thioglycollate or under anaerobic conditions, both of which have been reported as opposing the *in vitro* action of streptomycin,[2,3] is further evidence that these microorganisms were nonviable. Since Bacteroides, a common anaerobic saprophyte of the large bowel could be cultivated from most of these cases, even after *E. coli* had disappeared, it is possible that the Gram negative, non-motile, rods observed by direct microscopic examination belonged to this genus.

E. coli reappeared in the stool the day following the last dose of streptomycin. Fecal streptococci, Clostridia, Bacteroides and Candida were unaffected by the streptomycin in the stool. It is noteworthy that Candida appeared in the stool on occasions after *E. coli* had disappeared (Table I). No change in the character of the stool was observed during streptomycin administration. The color, consistency and quantity was the same during therapy as it had been before.

Discussion. The results obtained in this study are similar to those reported by Reimann, Price and Elias,[4] and others,[8] who found that sensitive microorganisms could be eliminated from the stool by oral administration of streptomycin; and that the stool could be kept free of such microorganisms as long as adequate streptomycin levels were maintained. These authors also noted that the anaerobic flora of the stool was unaffected by oral streptomycin.

It is of interest that although *E. coli* was eliminated from the stool in 3 cases of ulcerative colitis, no apparent effect upon the course of the disease was noted. In order to determine whether or not viable *E. coli* were present on the surface of the mucosa of the colon, in the cases who received streptomycin preoperatively, direct swabs were taken at operation. In neither of these cases could *E. coli* be cultivated.

It is known that the non-absorbable sulfonamides will reduce the number of *E. coli* present in the stool. In some cases, however, no reduction has been observed, yet in spite of the failure of these drugs to eliminate *E. coli*, clinical reports indicate that pre-operative administration decreases the incidence of complicating peritonitis and abscess formation following surgical manipulation of the colon.[5,6,7] It would seem on the basis of this study, that streptomycin is more effective than the sulfonamides in ridding the colon of *E. coli*. It has the further advantage of avoiding the risks associated with the use of the sulfonamides. Accordingly, the oral administration of streptomycin in the pre-operative preparation of patients who are to undergo surgery of the colon is suggested.

Summary. The oral administration of as little as 1.0 g of streptomycin daily eliminated *E. coli* from the stool of 5 patients within 2 days. The stool could be kept free of these microorganisms as long as adequate streptomycin levels were maintained, but reappeared promptly when it was discontinued. Swabs taken at operation from the mucosa of the colons of two patients who had received preoperative streptomycin did not contain *E. coli*. These microorganisms also were eliminated from the distal segment of a colostomy by streptomycin lavage per rectum. Streptomycin did not affect the anaerobic flora of the stool, and had no appreciable effect on the fecal streptococci.

2 Donovick, R., and Rake, G., PROC. SOC. EXP. BIOL. AND MED., 1946, 61, 224.

3 Geiger, W. B., Green, S. R., and Waksman, S. A., PROC. SOC. EXP. BIOL., AND MED., 1946, 61, 187.

4 Reimann, H. A., Price, A. H., and Elias, W. F., Arch. Int. Med., 1945, 76, 269.

5 Zintal, H., Lockwood, J. S., and Snyder, J., Bull. Am. Coll. Surg., 1943, 28, 51.

6 Behrend, M., Surg. Clin. North Am., 1944, 24, 238.

7 Bacon, H. E., et al., J. Internat. Coll. Surg., 1945, 8, 20.

8 Pulaski, E. J., and Amspacher, W. H., Bull. U. S. Army M. Dept., 1946, 6, 750.

16036 P

Effect of Penicillin on the Reaction Between Phage and Staphylococci.

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In Fleming's original paper on penicillin,[1] he described the lysis of staphylococci by this agent. Since then the lytic process and ancillary morphologic changes in the bacteria concerned have been studied by several investigators.[2-8] We wish to report here the effect of penicillin on the lytic action of bacteriophage.*

The phage employed in our experiments is the "K" race; it and the homologous strain of *Staphylococcus aureus* have been used in studies described in a series of papers appearing since 1929. Stock cultures were grown in Roux flasks for 18 hours at 36°C. In order to have a substrate of actively growing cells, 1×10^8 staphylococci/ml were suspended in 100 ml of broth contained in a 500 ml flask; aeration was provided by placing the flask in a shaker operating in a water bath set at 36°C. When growth had progressed to a level of 1×10^9 bacteria/ml, the cell suspension was removed from the shaker and used for the experiments described below. To determine [bacteria] when values were $>5 \times 10^8$ staphylococci/ml, the direct microscopic count method was employed. For lytic curves, visual comparison was made with standards covering a range from 2×10^7 to 5×10^8 staphylococci/ml in formalinized broth. The fluid medium throughout was tryptose phosphate broth. Phage determinations were carried out by Gratia's method[9] of counting plaques and the values noted below are in plaques/ml.

A 10 ml mixture containing 5×10^8 staphylococci/ml, 5×10^8 phage units/ml and 10 units of sodium penicillin/ml was placed in a test tube and shaken at 36°C. Turbidity readings were made every 0.2 hour. This was also done with a suspension from which the phage was omitted and with one containing no penicillin. Using as an end-point the reduction of turbidity by 50%, i.e., to 2.5×10^8 staphylococci/ml, it was observed that the suspensions lysed in the following order: Phage + penicillin, 0.8 hours; phage only, 2.0 hours; penicillin only, 2.7 hours.

When the initial concentration of phage was reduced to 5×10^7 phage units/ml, the respective times of lysis were: Phage + penicillin, 1.1 hours; phage only, 3.8 hours.

The accelerating effect could not be demonstrated in suspensions made up in Locke's solution; lysis was considerably delayed in the phage + penicillin suspension and the lytic curve paralleled that for penicillin alone.

Repetition of the experiment with concentrations of penicillin varying from one unit/ml to 1,000 units/ml showed that the acceleration of lysis took place uniformly in all concentrations and was independent of (penicillin) within this range, 0.01 unit of penicillin/ml and lesser concentrations had no measurable effect in speeding up phage-engendered lysis.

An experiment was performed to determine how long a period of penicillin action was required in a mixture of bacteria and phage

1 Fleming, A., *Brit. J. Exp. Path.*, 1929, **10**, 1.

* Since preparation of this article for publication, Dr. Winston Price of Rockefeller Institute has informed us in a personal communication of similar studies he has completed using another phage and bacterial substrate.

2 Gardner, A. D., *Nature*, 1940, **146**, 837.

3 Fleming, A., *Lancet*, 1941, **241**, 781.

4 Smith, L. D., and Hay, T., *J. Franklin Inst.*, 1942, **233**, 598.

5 Weiss, L. J., *Proc. Indiana Acad. Sci.*, 1943, **52**, 27.

6 Miller, C. P., and Foster, A. Z., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 205.

7 Todd, E. W., *Lancet*, 1945, **248**, 74.

8 Fisher, A. M., *J. Bact.*, 1946, **52**, 539.

9 Gratia, A., *Ann. Inst. Pasteur*, 1936, **57**, 652.

to produce the characteristic acceleration. Several 9 ml mixtures of phage and bacteria in test tubes were prepared containing 5 x 10^S staphylococci/ml and 2.5 x 10^S phage units/ml. The tubes were placed in the shaker at 36°C and at intervals of 10, 20, 40, and 60 minutes from the time of mixing, one ml of penicillin solution (10 units) was added to successive tubes.

The addition of penicillin at 10 minutes and 20 minutes produced 50% lysis 0.7 hours ahead of the tube containing phage only. When penicillin was added after 40 minutes had elapsed, the lytic end-point occurred 0.5 hours before that in the phage control. With these particular concentrations of bacteria and phage, a typical acceleration could be secured if the penicillin acted at least 0.9 hours on the cellular substrate. Exposure to penicillin for a period of from 0.4 to 0.7 hours reduced the time of lysis by 0.5 hours.

In many of the experiments performed when the initial concentrations of bacteria were 5 x 10^S staphylococci/ml or greater, the initial phage concentration 5 x 10^S phage units/ml and the concentration of penicillin between 10 and 1,000 units/ml, clearing of the suspensions occurred without any increase in turbidity. Despite the absence of obvious bacterial reproduction, there were increases in [phage], up to a maximum of 10-fold, as determined by the plaque count. The methods used for detecting any increase in [bacteria] were not critical, however, and this point should be reinvestigated with more sensitive procedures.

A detailed account of further experiments with penicillin and phage action will be published elsewhere.

16037

A Bacteriophage for *Mycobacterium smegmatis*.[*]

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During investigations on the isolation of microorganisms antagonistic to the mycobacteria a bacteriophage active for *Mycobacterium smegmatis* was encountered.[1] The finding of this bacteriophage is of interest because it is, to our knowledge, the first definite evidence of a bacteriophage among the mycobacteria. Steenken[2] described spontaneous lysis in old colonies of *Mycobacterium tuberculosis* H37Rv which may have been due to bacteriophage action. He determined that the lytic factor was filterable but did not identify it as a bacteriophage by all of the usual criteria. Lysis appeared when the cultures were 3 to 4 months old and the pH of the medium had dropped to values of about 4.2 to 4.6. It began in the center of the colonies and spread to the periphery. The filtrate produced lysis of living bacteria at pH 4.2 to 4.8 and of both living and heat-killed bacteria at pH 2.2 to 4.0. Secondary resistant colonies developed in old liquefied areas.

The fact that bacteriophages for the mycobacteria have not been reported previously suggests that they may be very limited in their distribution. The successful isolation of one of them in the present work may have been due to the particular enrichment treatment employed.

The enrichment was carried out on 200-g samples of moist leaf compost to which a small amount of calcium carbonate was added. The

1 Weiser, R. S., and Gardner, G. M., unpublished results.

2 Steenken, W., *Am. Rev. Tub.*, 1938, 38, 777.

samples were collected from various locations in the city. They were incubated at 37°C for 8 months and treated semi-weekly with 5 to 10 ml of a heavy well-washed suspension of a young culture of *M. smegmatis*. The suspension was prepared from organisms grown on glycerine broth for one week at 37°C. The culture mass was ground in a mortar and washed twice in physiological salt solution.

After 3 months of enrichment, tests for organisms antagonistic for *M. smegmatis* were begun by making fixation plates of dilutions of the compost ranging from 1:100 to 1:100,000. The medium used for plating was nutrient agar containing 1% glycerine and heavily inoculated with *M. smegmatis*.

Soon after beginning the tests, bacteriophage plaques were noted on the plates made from two of the compost samples. They had smooth edges and displayed a halo of partial lysis about a central clear zone of complete lysis. Isolated plaques attained a diameter of about 3 mm. Smears made from the clear areas contained only an occasional acid-fast organism. The material from the clear areas, when sub-cultured on bacteriological media, likewise, yielded only acid-fast organisms.

A Berkefeld filtrate was prepared from plaque material and the bacteriophage sub-cultured several times on plates. Filtrates of plaque material from the sub-cultures contained the bacteriophage in a concentration of 300 billion particles per ml.

No particular attempt was made to determine that the bacteriophage was a pure strain. However, preliminary tests indicate that the bacteriophage is specific for our stock strain of *M. smegmatis*. It proved to be inactive for *Mycobacterium phlei* and a second strain of *M. smegmatis*.

The bacteriophage was inactivated by a temperature of 75°C for 10 minutes but remained active when held at 72°C for 10 minutes. It preserved well in 50% glycerine and by lyophilization.

As a test of the effectiveness of the method used for the isolation of bacteriophage for *M. smegmatis* the enrichment procedure was repeated using a sample of soil collected on the University campus. Precautions were taken in this trial to prevent possible contamination of the test plates with the specific bacteriophage we had previously isolated. Weekly tests for the presence of bacteriophage were negative until the third week of enrichment when plaques first appeared. Tests on the control unenriched sample of soil were negative for bacteriophage. The plaque characteristics of this newly isolated bacteriophage did not appear to be different from those of the previous isolate.

Discussion. In our work on the isolation of microorganisms antagonistic to the mycobacteria we have used both enriched and unenriched soil and compost. The bacteriophage was isolated from 2 of 6 samples of enriched compost and from one sample of enriched soil. It was not encountered in 8 samples of unenriched compost and 4 samples of soil. Apparently the isolation of the bacteriophage was facilitated by the specific enrichment employed, and possibly because the enrichment was carried out on the soil and compost samples preliminary to filtration rather than after filtration.

Bottcher and Hofer[3] have employed specific preliminary enrichment of soil for the isolation of bacteriophage for the legume bacteria by adding a special medium heavily inoculated with the organism. The present method differs from that of Bottcher and Hofer inasmuch as the organisms added were free of medium. It is possible that the lack of medium may operate to advantage by reducing the possibility of suppression of the specific organism or its bacteriophage by overgrowth of some other organism.

Summary. A bacteriophage specific for *M. smegmatis* was isolated from samples of compost and soil by specific enrichment with a heavy washed suspension of *M. smegmatis*. The method of enrichment employed may be useful in the isolation of other phages.

3 Bottcher, J., and Hofer, A. W., *J. Bact.*, 1943, 45, 407.

16038

Tissue Toxicity of the Germicides Iodine and Bromine.[*]

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Lambert[1,2] as early as 1916, defined the ideal antiseptic as one which kills the infecting agent without causing injury to body cells. In accordance with this definition he evaluated a series of germicides in terms of relative toxicity to *Staphylococcus aureus* and human connective tissue cells respectively. In different later investigations, various other tissues were used for tests of this type. Lampert and Meyer[3] used rabbit spleen, German[4] and Buchsbaum and Bloom[5] used chick tissue culture, while Salle and Lazarus[6] used embryonic chick heart tissue for their experiments. In experiments carried out by Salle and McOmie[7] and Salle, McOmie and Schechmeister,[8] embryonic tissue was used as the test substance. Similar investigations were carried out by Osgood[9] and Herrell and Heilman.[10]

In other methods of determining toxicity for tissue, living chick embryos have been employed as the test object. The living embryo is preferable to tissue culture for this purpose as it presents conditions which approach more closely the complexity of the tissue interrelations of the intact animal. The developing egg presents the additional advantage that it consists of rapidly growing embryonic tissue in a perfect nutritional environment, and furthermore contains large amounts of albuminous material with which the bactericidal agent can come into contact. Moreover, the chick embryo is convenient to handle because of mechanical protection and the ease of repeated access to the embryo which is afforded by the egg shell.

Tests of disinfectants for their potency *in vivo* by chick embryo methods, were carried out by Witlin,[11] Dunham,[12] and Green and Birkeland.[13]

In a third method the toxicity test is carried out in the presence of blood. The use of this test object is indicated because of its known role in infection, and because of the high sensitivity of blood cells to the germicides. Welch and Brewer[14] claim that "application of antiseptics which destroy this function at dilutions which cannot destroy bacteria is a harmful practice". In papers by Nye,[15] Welch and Hunter,[16] and Hirsh and Novak,[17] phagocytes were used as test object of the toxicity assays.

In the following experiments the two last

1 Lambert, R. A., *J. Exp. Med.*, 1916, 24, 683.

2 Lambert, R. A., *J. A. M. A.*, 1916, 67, 1300.

3 Lambert, R. A., and Meyer, J. R., PROC. SOC. EXP. BIOL. AND MED., 1926, 23, 429.

4 German, W. J., *Arch. Surg.*, 1929, 18, 1920.

5 Buchsbaum, R., and Bloom, W., PROC. SOC. EXP. BIOL. AND MED., 1931, 28, 1060.

6 Salle, A. J., and Lazarus, A. S., PROC. SOC. EXP. BIOL. AND MED., 1935, 32, 1481; 33, 8, 665, 937, 1057, 1119.

7 Salle, A. J., McOmie, W. A., and Schechmeister, I. L., *J. Bact.*, 1937, 34, 267.

8 Salle, A. J., McOmie, W. A., and Schechmeister, I. L., PROC. SOC. EXP. BIOL. AND MED., 1938, 37, 694.

9 Osgood, E. E., *Arch. Int. Med.*, 1938, 62, 181.

10 Herrell, W. E., and Heilman, D., *Am. J. Med. Sci.*, 1943, 205, 157.

11 Witlin, B., PROC. SOC. EXP. BIOL. AND MED., 1942, 49, 27.

12 Dunham, W. B., PROC. SOC. EXP. BIOL. AND MED., 1942, 50, 274.

13 Green, T. W., and Birkeland, J. M., PROC. SOC. EXP. BIOL. AND MED., 1942, 51, 55.

14 Welch, H., and Brewer, C. M., *J. Immunol.*, 1942, 43, 25.

15 Nye, R. N., *J. A. M. A.*, 1937, 108, 280.

16 Welch, H., and Hunter, A. C., *Am. J. Pub. Health*, 1940, 30, 129.

17 Hirsch, M. M., and Novak, M. V., PROC. SOC. EXP. BIOL. AND MED., 1942, 50, 376.

TABLE I.
Effect of Halogens on Phagocytosis.

Exp. No.	Halogen	Concentration, %	Polynuclears taking part in phagocytosis, %	No. of bacteria ingested per 100 cells
1	Br_2	1.75	45.7	1.2
	I_2	0.5	13.5	0.27
2	Br_2	1.25	52.0	1.76
	I_2	0.25	25.5	0.57

mentioned methods were used with some modifications.

In earlier tests comparing the disinfectant activities of bromine and iodine, it was observed that bromine *in vivo* (rabbit test) was more active than iodine, whereas the latter was more effective than bromine as a disinfectant *in vitro* (Rideal-Walker test). This suggested that bromine applied on living tissue might be a more efficient antiseptic for use on wounds, cuts and abrasions than iodine.

Toxicity tests on both halogens were undertaken. The method of Hirsh and Novak[17] was employed. In this method the highest dilution which destroys the phagocytic power of leucocytes is determined (toxicity end point) and divided by the highest dilution which kills the test organism (germicidal end point) under the same conditions. Thus, by dividing both end points a toxicity index which expresses the disinfectant power of the tested substance *in vivo* is obtained. A difficulty was encountered in carrying out the test as described by Hirsh and Novak.[17] Even when 7 parts of saturated bromine solution of 3.5% were added to one part of blood marked phagocytosis still occurred. Thus, when the prescribed amount of blood was decreased from 50% to 12.5%, the toxicity end point was still not reached. It is clear, however, that the toxicity index is well under 1, and therefore highly favorable.

In another series of experiments a slight modification of this experimental technique was employed. The germicidal end point was determined as described above, but the harmful effect of the halogens on the leucocytes was evaluated by making blood smears after contact of the blood cells with the halogens for 30 minutes at 37°C in dilution

which prevents growth of the test organism. The bacteria were added and a contact time of 10 minutes was allowed, the mixture being shaken thoroughly in the meantime in a water bath of 37°C to assure contact between blood cells and bacteria. After staining the blood smears with Loeffler's methylen blue, 100 polymorphonuclear leucocytes were counted and (1) the number of the cells taking part in phagocytosis and (2) the number of bacteria ingested were determined. By comparing the effect of the 2 halogens, the one more harmful to the leucocytes could be recognized. The results are summarized in Table I.

In a further series of experiments, a modification of Green and Birkeland's[13] method for testing a disinfectant on living embryo was used. Chorio allantois of living embryos was infected with S. aureus according to Goodpasture and Buddingh.[18] The amount of bacteria was so chosen that the embryo was killed after 24-48 hours. When disinfectant is added in an appropriate amount, it stops the development of the bacteria, and the embryo can survive the infection. The disinfectant to be tested is used in a concentration which in blank experiments does not have a harmful effect on the embryo. After infecting the chorio allantois with a lethal dose of bacteria, a specific time is allowed to enable the bacteria to begin logarithmic growth. The proper time was determined by agar plate counts. The disinfectant to be tested is added about 3-4 times during 48 hours. By comparing the death rate occurring after treatment with various disinfectants, the relative efficacies were determined.

18 Goodpasture, E. W., and Buddingh, G. J., *Am. J. Hyg.*, 1935, 21, 319.

TABLE II.
Antibacterial and Toxic Action of Halogens on Living Chick Embryos.

Bacteria inoculated	Halogen	Conc., %	No. of eggs under exper.	No. of dead embryos after 48 hr	Death rate, %
2x10⁶	—	—	23	20	87
2x10⁶	Br₂	0.2	11	5	45
2x10⁶	Br₂	0.06	12	6	50
2x10⁶	I₂	0.08	16	14	87.5
—	I₂	0.08	5	1	20
—	Br₂	0.1	5	0	0
—	Br₂	0.06	5	1	20

In each experiment 2x10⁶ cells of *S. aureus* from 18-24-hours-old broth culture were used. The eggs were placed for 2-3 hours in an incubator at 37°C to allow the bacteria to begin to multiply. The disinfectants were added after 3, 8, and 24 hours. In control experiments saline instead of halogens was added. The embryos were opened after 48 hours. The results obtained with bromine and iodine are given in Table II. These experiments, too, show that bromine in presence of living tissue is a better disinfectant than iodine.

Conclusion. The assertions of Babcock[19] concerning the effectiveness of bromine *in vivo* in treatment of wounds is confirmed by the toxicity tests.

19 Babcock, W. W., *J. A. M. A.*, 1945, **129**, 1094.

16039

Mumps Meningo-Encephalitis. Isolation in Chick Embryos of Virus from Spinal Fluid of a Patient.[*]

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Meningo-encephalitis may occur as a complication of parotitis or as a primary manifestation of infection with the virus of mumps. Whereas in the first instance the time relationships tend to permit a diagnosis of mumps meningo-encephalitis, the diagnosis of the primary encephalitic manifestation has been placed on a firm basis only since the development of a complement-fixation technic by Enders and his co-workers.[1] The demonstration of a rise in complement-fixing antibodies in convalescent serum as compared to the antibody titer in the serum specimen obtained in the first days of the disease may be taken as diagnostic evidence of mumps.[2] By this means, a considerable number of cases of meningo-encephalitis without prior or concomitant parotitis can be diagnosed as due to mumps.[3] Differentiation between antibodies to the soluble and to the virus antigens[4] may be of further diagnostic help.

1 Enders, J. F., Kane, L. W., Cohen, S., and Levens, J. H., *J. Exp. Med.*, 1945, 81, 93.

2 Enders, J. F., Cohen, S., and Kane, L. W., *J. Exp. Med.*, 1945, 81, 119.

3 Kane, L. W., and Enders, J. F., *J. Exp. Med.*, 1945, 81, 137.

4 Henle, G., Henle, W., and Harris, S., PROC. SOC. EXP. BIOL. AND MED., 1947, 64, 290.

Antibodies to the soluble antigen tend to appear earlier than antibodies to the virus antigen in a high percentage of infections with the mumps virus. When this pattern of serum reactivity occurs, it is only found in the first few days of infection.[5]

Another approach to the diagnosis of mumps meningo-encephalitis has been the attempt to isolate the etiological agent from the spinal fluid. One such attempt has been successful in the monkey[6] but inoculation of chick embryos failed.[6,7] In the case to be reported here a virus was obtained from the spinal fluid of a patient with meningo-encephalitis without parotitis on the second day of illness by the intra-amniotic inoculation of chick embryos. This agent has been identified as mumps virus.

Case history. The 5½-year-old patient A.P. was admitted to the hospital on 17 March 1947, complaining of fever, severe headache, and occasional vomiting of 2 days duration. The child appeared moderately ill. Physical examination revealed mild nuchal rigidity but no other neurological signs. A slight injection of the pharynx and of the orifices of Stenson's ducts was noted but no parotid or other glandular swelling. The temperature on admission was 103°F, the pulse 108 per minute. All other examinations were negative.

In the night following admission the temperature rose to 105°F, but thereafter decreased steadily and returned to normal on the 4th day in the hospital. The headache and nuchal rigidity persisted for 2 days. On the day after admission, 18 March 1947, a slight disturbance in swallowing was reported which was of very short duration, and complaints of moderate abdominal pain were reported. Thereafter the child improved steadily and was released from the hospital after 15 days on 1 April 1947. There was no known exposure of the patient to mumps. However, a sister developed parotitis on 29 March 1947.

Laboratory findings. Spinal fluid was obtained from the patient on the day of admission, *i.e.*, on the second day of illness. The initial pressure was 450 mm H_2O, the final recording 290 mm. The fluid was slightly cloudy and contained 450 cells per cu mm, of which 92% consisted of lymphocytes, 7% of endothelial cells, and 1% of polymorphonuclear leukocytes. No bacteria could be demonstrated in stained smears, nor could any organisms be cultured on bacteriological media. The total protein in the fluid was 10 mg, sugar, 30 mg and chlorides 712 mg/100 ml. Subsequent spinal fluids taken 24 March and 31 March 1947 showed similar, although increasingly milder, changes, the last specimen containing 55 cells per cu mm.

The blood picture on admission was as follows: 4,500,000 erythrocytes, 13 g hemoglobin, 17,000 white cells, of which 60% were granulocytes, 40% lymphocytes, and 4% monocytes. A subsequent study of the blood on 31 March 1947 was essentially normal.

Other studies included tuberculin tests with O.T. 1:10,000 and 1:1,000, which were negative. The Kolmer-Wassermann, Kahn and complement-fixation tests for lymphocytic choriomeningitis with the serum of the patient taken 1 April 1947, likewise, were negative. Attempts to isolate this virus from the spinal fluid drawn 17 March 1947 failed.[†]

Serological tests for mumps. The preparation of complement-fixation antigens and the technic used for the complement-fixation test have been described.[4] The tests performed with the sera obtained during the early stage of the disease were not revealing in this case.

TABLE I.
Result of Complement Fixation Tests with Sera from Patient A.P.

Serum specimen	Day after onset of disease	Serum titer (initial dilution) vs.	
		Soluble antigen	Virus antigen
3-17-47	2	1: 4*	1: 4
21	6	1: 4	1: 8
25	10	1: 8	1: 32
4- 1	17	1:16	1:128

* Complete fixation of complement in serum dilution 1:4.

† These tests were performed by Dr. M. M. Sigel.

5 Henle, G., to be published.

6 Swan, C., and Mawson, J., *Med. J. Austral.*, 1943, **1**, 411.

7 Beveridge, W. I. B., Lind, P. E., and Anderson, S. G., *Austral. J. Exp. Biol. and Med. Sci.*, 1946, **24**, 15.

As can be seen in Table I, low concentrations of antibodies to the soluble as well as to the virus antigen were found on the 2nd day after onset. However, a definite diagnosis of infection with mumps virus was possible because subsequent specimens of serum showed significant rises in antibodies to both antigens.

Isolation of virus. Spinal fluid, drawn on the second day of illness was kept frozen at −10°C until injection of chick embryos was possible. Eight 8-day-old embryos were inoculated into the amniotic sac with 0.1 ml of undiluted spinal fluid each. After incubation of the eggs at 36 to 37°C for 5 days, the amniotic and allantoic fluids of each embryo were collected separately and tested on a slide for their capacity to agglutinate chicken red cells.[s] A slight degree of hemagglutination was observed with several of the amniotic fluids. The bacteriologically sterile pool of all amniotic fluids was used for further passage. Five out of 16 embryos of the second amniotic passage died, and of the 11 remaining embryos 10 amniotic fluids but none of the allantoic fluids showed positive hemagglutination. On the 4th amniotic passage the virus in the amniotic fluid reached a 50%-infectivity end point of $10^{-6.3}$ and a hemagglutinin titer of 1:1024 as measured by the pattern test.[4] From the 7th amniotic passage on, when the hemagglutinin titer in the amniotic fluid exceeded 1:4096, a slight degree of hemagglutination became discernible in the allantoic fluids. Inoculation of amniotic fluid of the 9th passage into the allantoic sac of 8-day-old chick embryos resulted in some propagation of the agent in this cavity. After 5 days of incubation, the pooled allantoic fluids showed a hemagglutinin titer of 1:128.

The identity of the virus has been established in 3 ways. First, neutralization tests were performed in chick embryos with amniotic fluid of the 4th passage. Rabbit anti-mumps serum in dilution 1:10 neutralized 10,000 ID_{50} of the agent, whereas rabbit anti-influenza sera failed to do so. A human convalescent serum of high complement-fixing activity reacted to an extent comparable to that of the specific rabbit immune serum. Second, complement-fixation tests with the amniotic fluid of the 5th amniotic passage as antigen revealed strong reactions with known mumps convalescent sera but no, or lesser, reactions with sera taken from the corresponding patients during the acute stage of the disease. Finally, the same amniotic fluid (5th passage) was found to produce typical, although mild, parotitis in 2 out of 4 individuals who were judged susceptible to mumps by the results of complement-fixation tests prior to exposure.[‡] All 4 cases developed antibodies to both the soluble and virus antigens. Thus, there was no doubt that the agent constituted a strain of mumps virus. This strain differed markedly from the one present in the laboratory at that time in the following respects: The new strain grew in the early passages only in the amniotic sac whereas the laboratory strain propagated equally well in the allantoic and amniotic cavities; also, the new strain gave rise to clinical mumps in exposed human subjects, while the laboratory strain produced only subclinical infections as demonstrable by rises in antibodies beginning on the 13th to 19th day after exposure.[‡]

Summary. A virus has been isolated from the spinal fluid of a patient with meningo-encephalitis without parotitis by inoculation of chick embryos by the amniotic route. This agent has been identified as mumps virus.

NOTE: Since this paper was written mumps virus has been recovered from the spinal fluid of another patient (T.P.) with meningo-encephalitis without parotitis. Positive red cell agglutination was obtained in second passage amniotic fluid. The finding of circulating complement-fixing antibodies against the soluble antigen (titer 1:16) but not against the virus antigen of the mumps virus established the diagnosis on the second day of illness.

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s Levens, J. H., and Enders, J. F., *Science*, 1945, 102, 117.

‡ These data, obtained in collaboration with Drs. Joseph Stokes, Jr., and Harriet Davis, will be published in greater detail separately.

16040 P

Effects of Radiotoxic Dosages of I^{131} upon Thyroid and Contiguous Tissues in Mice.*

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It seems remarkable that despite the extensive use of radioactive iodine in experimental and clinical studies no information has appeared in the literature concerning the radiation dosage from which injury may be expected in the thyroid gland. Such information seems implied in the use of I^{131} for the destructive irradiation of human thyroid carcinoma, but even here knowledge of a possible differential in radiation-sensitivity between normal and neoplastic thyroid tissue is greatly to be desired.

In an exploratory study, 36 inbred mice (A and C_{57} strains), 2 to 5 months old, and of both sexes, have been given "tracer" quantities of I^{131} in the form of NaI in neutral aqueous solution by subcutaneous injection. Mice were fed purina laboratory chow and kept at a constant temperature of $72° \pm 1°F$. Radiation dosages ranged from 100 to 1000 microcuries. Calculation of dosage was made from standardizations on a Geiger counter which, in a survey by the National Bureau of Standards in June, 1947, compared favorably in sensitivity to I^{131} with instruments at most other institutions. Mice were sacrificed 2, 3, 24, or 120 days after injection. Thyroids and surrounding tissues were serially sectioned.

Table I provides an approximate summary of the observations made.

A striking early effect of the localized radiation was a periglandular edema which separated the thyroid from surrounding structures and extended even into neighboring muscle. The edematous connective tissue was extensively infiltrated by lymphocytes, polymorphonuclear leucocytes, and some mast cells. Pycnosis of areas of tracheal epithe-

lium was common. In animals killed on the second day following injection of 300 microcuries (20-23 millicuries I^{131} per kg) there was little effect beyond this. In the animal killed 2 days after receiving 53 mc per kg the thyroid appeared as an eosinophilic mass, amorphous in medullary parts, but with recognizable surviving follicles in the peripheral parts of the gland. Cells with still stainable nuclei either were pycnotic or else showed signs of activity (hypertrophy, exhaustion of colloid). In all instances surviving thyroid tissue was in the isthmus or the cranial apex of the gland where, presumably, self-radiation would have been minimal.

On the third day after injection thyroid destruction varied from minimal with lower dosages to complete with the 50 mc per kg dose. Parathyroid involvement paralleled these changes. Furthermore, dosages above 20 mc per kg produced pycnosis of nuclei of neurilemma and sheath cells in the recurrent laryngeal nerve, in those sections most intimately associated with the thyroid.

After a 24 day exposure to I^{131} radiation even the low dose (3-5 mc per kg) produced extensive destruction, and a medium dose (18-22 mc per kg) produced complete thyroidal destruction. At this time fibrosis had replaced the amorphous mixtures of epithelial debris and leucocytes with a somewhat edematous type of fibrous tissue. No normal parathyroid tissue remained.

At the 120 day interval the thyroid consisted of a shrunken fibrous band, the edema and leucocytic reaction having disappeared. In animals having received the low dose of I^{131} most glandular tissue had disappeared but a few surviving small follicles were imbedded in the fibrous tissue, and seemed to be secreting actively, and possibly serving as a reservoir for regeneration.

TABLE I.
Summary of Results.

Exposure to I^{131}, days	No. of mice	Dose, millicuries per kg	Approx. % thyroid destruction	Approx. % parathyroid destruction
2	2	20-23	0	0
2	1	53	90	75
3	6	3-5	10	0
3	4	17-18	75	25
3	4	30-35	80-90	75
3	3	50-55	100	95
24	8	3-5	25-50	10
24	4	18-22	100	50
120	4	3-4	90	100

Summary. Dosages of I^{131} from 3 to 50 millicuries per kilogram were given to young mice on a normal diet. Higher dosages produced complete thyroidal destruction within a few days. Lower dosages permitted survival of some thyroidal epithelium in the isthmus and cranial apex of the thyroid for as long as 120 days after injection, but resulted in loss of the parathyroids. Lesions were noted in the tracheal epithelium with all dosages, and in the recurrent laryngeal nerve with higher dosages.

16041 P

Thiouracil and Conversion of Carotene to Vitamin A Measured by Liver Storage in the Rat.[*]

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In a recent report, Canadell and Valdecasas[1] fed rats, which previously were on a vitamin A-free diet with thiouracil administered in the drinking water, 60 γ of carotene per week. The administered carotene was unable to relieve the ocular symptoms produced by a vitamin A deficiency. However, these symptoms were alleviated if small amounts of thyroid powder were administered with the carotene or vitamin A was fed to the thiouracil-treated animals. Their interpretation of this phenomenon was that it involved an inhibition of carotenase.

If this is true, then the feeding of carotene to vitamin A deficient-animals previously treated with thiouracil should produce little or no vitamin A in the liver. Therefore, this experiment was attempted, and a preliminary report of the results obtained is presented.

Twenty-six rats were placed on a vitamin A-low diet when they were 10 days old. When they reached a body weight of 43 g, they were divided into 2 groups. One group was continued on the same diet for 4 weeks while the second group received a similar diet to which 0.25% of thiouracil had been added. All rats were continued for an additional 2 weeks on identical diets except that they were made vitamin A-free by replacing the commercial casein with vitamin A-free test casein

1 Canadell, J. M., and Valdecasas, F. G., *Experientia*, 1947, 3, 35.

TABLE I.
Body Weight and Vitamin A Content of Young Rats Receiving a Vitamin A-free Diet With or Without Thiouracil at Periods of 36 Hours to 7 Days After Feeding 348 γ of β-carotene.

Carotene fed, γ	Rats administered diet containing 0.25% thiouracil			Control rats receiving no thiouracil		
	No. of rats	Body wt, g	Vit. A content per liver,* I.U.	No. of rats	Body wt, g	Vit. A content per liver,* I.U.
0	6	83	(4.5)†	6	156	(6.7)†
348	8	86.5	56.7 ± 24.4 (11.4-232)‡	6	143	56.2 ± 10.1 '(17.9-91.5)‡

* Includes standard error of the mean calculated as follows:

$$\sqrt{\Sigma d^2/n} \,/\, \sqrt{n}$$

where "d" represents the deviation from the mean and "n" is the number of observations.
† Not considered as vitamin A since no fading blue color developed.
‡ Range of results.

(General Biochemicals, Inc.). Some of the animals in each group received a single oral dose of a solution of β-carotene in cottonseed oil containing 348 γ while the remaining rats, which served as controls, received cottonseed oil. The animals were sacrificed at 36 hours, 5 days or 7 days. Since the vitamin A levels in the different groups did not show any significant trend with time, the results obtained with animals sacrificed at 36 hours, 5 or 7 days have been averaged together. Vitamin A was extracted from the livers by the procedure employed by Mattson, Deuel and Mehl.[2] The determination of vitamin A was by the Carr-Price method using a Coleman Junior spectrophotometer. The results are summarized in Table I.

It would appear from these experiments that a thiouracil-treated rat can convert carotene to vitamin A and store this product in the liver when fed 348 γ of carotene. However no results on the animals' ability to utilize this stored vitamin A are presented. Drill and Truant[3] in a recent paper, using thyroidectomized animals, could not prevent or alleviate ocular symptoms by injecting 10 γ of carotene per day. However, Remington et al.[4] found that an oral dose of 0.6 γ of carotene per day was able to bring about a cure of the eye symptoms of thyroidectomized rats within 7-9 days. No difference between the effectiveness of vitamin A and carotene was noted in these experiments.

Experiments are now under way to demonstrate the lowest level of carotene and vitamin A which must be fed to a thiouracil-treated animal to alleviate the eye symptoms from vitamin A deficiency.

2 Mattson, F. H., Mehl, J. W., and Deuel, H. J., Jr., Arch. Biochem., 1947, 15, 65.

3 Drill, V. A., and Truant, A. P., Endocrinology, 1947, 40, 259.

4 Remington, R. E., Harris, P. L., and Smith, C. L., J. Nutrition, 1942, 24, 597.

16042

Antipyridoxine Activity of Methoxypyridoxine in the Chick.

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Desoxypyridoxine (2,4-dimethyl-3-hydroxy-5-hydroxymethylpyridine), an analogue of pyridoxine, has been shown to be a strong inhibitor of pyridoxine in chicks[1] and to exhibit a similar but less potent effect in rats.[2] Further studies on additional analogues of this vitamin have led to the discovery that 2-methyl-3-hydroxy-4-methoxymethyl-5-hydroxymethylpyridine (methoxypyridoxine) also has pronounced antipyridoxine activity in chicks. In rats, however, this methoxypyridoxine has some vitamin activity,[3] presumably due to the ability of this species to convert part of the compound to pyridoxine.[4]

The procedure employed in studies on the effect of methoxypyridoxine in chicks was similar to that described previously.[1] To begin an assay, eight-day-old chicks weighing approximately 60 g after maintenance for 5 days on a purified diet (Table I) deficient in pyridoxine were distributed into groups balanced in regard to body weight. The chicks were dosed orally with the test substances on the first, third, fifth and seventh days of the assay period. Body weights were recorded on these days as well as on the ninth (last) day of the test. The pyridoxine-deficient diet was fed to all groups during the assay period.

The curve of response (weight gain *vs.* log dose) was established for each assay by using 3 or more of the groups of chicks on dosages in the range from 5 to 50 micrograms pyri-

TABLE I.
Pyridoxine-deficient Diet for Chicks.

	g
Dextrose (cerelose)	51.5
Casein (vitamin free)	25.0
Salts IV5	5.0
Cellulose (ruffex)	5.0
Calcium gluconate	2.5
Glycine	2.0
Liver extract L*	2.0
KH_2PO_4	1.0
Wheat Germ Oil	4.5
400 D fish liver oil	0.5
Arginine	0.5
Cystine	0.2
Choline	0.2
Inositol	0.1
p-Aminobenzoic acid	0.03
Niacin	0.01
Calcium pantothenate	0.004
Riboflavin	0.002
Thiamine	0.002
Menadione	0.0004
Biotin	0.00004
Total	100.05

* Wilson & Co., Inc., Chicago, Ill.

doxine per dose. The growth response of each group receiving a combination (premixed solution) of methoxypyridoxine and pyridoxine was compared with the curve of response for pyridoxine to determine the net pyridoxine activity of the combination. The inhibition ratio between analogue and vitamin was then calculated from the amount of methoxypyridoxine administered and the apparent loss in pyridoxine activity.

A single dose of 200 μg of methoxypyridoxine killed all the chicks in the group within 48 hours after administration (Table II). Even quantities as low as 15 μg per chick were fatal in the absence of pyridoxine. This toxic effect was prevented by simultaneous administration of an equal amount of pyridoxine. thus indicating that methoxypyridoxine had antipyridoxine activity. The bioassays (Table II) showed that approximately 4 parts by weight of methoxypyridoxine hy-

1 Ott, W. H., PROC. SOC. EXP. BIOL. AND MED., 1946, 61, 125.

2 Emerson, G. A., unpublished data.

3 Unna, K., PROC. SOC. EXP. BIOL. AND MED., 1940, 43, 122.

4 Porter, C. C., Clark, I., and Silber, R. H., J. Biol. Chem., 1947, 167, 573.

5 Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., J. Biol. Chem., 1941, 138, 459.

TABLE II.

Antipyridoxine Activity of Methoxypyridoxine in 8-day Curative Assays with Chicks Receiving a Pyridoxine-deficient Diet.

Exp.	Oral Supplement*		Survival of chicks, alive/total	Wt gain per chick, g	Loss in pyridoxine activity, μg	Ratio of inhibition, analogue : vitamin
	Pyridoxine hydrochloride, μg	Methoxypyridoxine hydrochloride, μg				
1	0	200	0/6	†		
	0	0	4/7	11.4		
	20	0	7/7	50.9		
2	0	15	0/7	‡		
	0	0	4/7	10.0		
	7.5	0	2/7	34.7		
	15	0	7/7	38.8		
	50	0	7/7	46.7		
	15	15	7/7	43.3	0	—
	15	30	5/7	33.5	8	4:1
	15	45	5/7	28.1	11	4:1
3	20	40	1/7	—	20	2:1
	20	60	5/7	20.8	16	4:1
	20	80	5/7	22.9	15	5:1
	0	0	6/7	14.5		
	5	0	14/14	24.9		
	10	0	13/14	31.6		
	20	0	6/7	37.1		
	40	0	7/7	56.2		
4	60	60	7/7	36.6	24	2.5:1
	60	120	3/7	26.0	42	3:1
	60	240	6/7	17.5	50	5:1
	60	360	3/7	8.7	55	6.5:1
	0	0	3/7	4.0		
	10	0	14/14	17.7		
	40	0	14/14	38.6		

* Amount given per chick on each of 4 successive alternate days.
† All dead after 1st dose.
‡ 2 dead after 1st dose, 3 dead after 2nd dose, 2 dead after 3rd dose.

drochloride counteracted the biological activity of one part by weight of pyridoxine hydrochloride. In the chick, therefore, methoxypyridoxine is approximately as powerful an inhibitor of pyridoxine as is desoxypyridoxine.[1]

The inhibition ratio of 2:1 reported for desoxypyridoxine[1] is not significantly different from the inhibition ratio of 4:1 estimated above for the methoxy analogue of pyridoxine. Nevertheless, there seems to be a difference in the antivitamin action of the two analogues. Although both compounds appear to compete with pyridoxine, it has been observed on the basis of limited tests that the effects of lethal doses of desoxypyridoxine were relatively easily counteracted by administration of pyridoxine even after a con-

siderable length of time. On the other hand, when a lethal dose of methoxypyridoxine had been given, subsequent administration of pyridoxine was generally ineffective in preventing the death of the chicks. These and other observations on comparative antipyridoxine activity in the chick are being investigated further.

Summary. 2-Methyl-3-hydroxy-4-methoxymethyl-5-hydroxymethylpyridine (methoxypyridoxine) has been shown to have the same order of antipyridoxine activity as desoxypyridoxine in chicks. In these experiments, approximately four moles of methoxypyridoxine counteracted the vitamin activity of one mole of pyridoxine when suboptimal or optimal amounts of the vitamin were given to pyridoxine-deficient chicks.

16043

Estimation of Alterations of Serum Gamma Globulin by a Turbidimetric Technique.

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Increased attention has been devoted in recent years to the elevation of serum globulin encountered in patients with diseases of the liver. The wide use of the cephalin flocculation and the thymol turbidity reactions for the diagnosis of liver disease has been partially responsible for this renewed interest. These reactions have been shown to depend mainly on small changes in the globulin fraction of the serum.[1,2] However, other factors are involved and the exact aberration of the serum that is measured has not been completely identified. In the case of the thymol turbidity test, for example, the concentration of the serum lipids has a definite effect on the intensity of the reaction.[2] It would seem important, therefore, to determine specifically and directly slight elevations of globulin that occur in acute liver disease.

The usual methods of globulin estimation which depend on nitrogen determinations following salting out procedures are subject to considerable error. The technique is time consuming and minor changes in the globulin level may not be apparent. Changes in the amount of serum globulin can best be detected at the present time by means of electrophoretic patterns. Such a technique, however, is not practical for routine application.

Dilution of serum with solutions of low ionic strength decreases the solubility of the proteins and under certain conditions the more insoluble globulins will precipitate. This technique is utilized in the thymol turbidity test[3] and in the buffer dilution test of Wolff.[4]

Both of these reactions are positive in acute liver disease and appear to depend partly on increases in the gamma globulin fraction of the serum. In certain conditions, such as cirrhosis of the liver, large increases in gamma globulin may be present in the serum in the absence of increased values for these two tests. The intensity of these reactions, therefore, is not always proportional to the increase in amount of gamma globulin in the serum.

The present paper describes a turbidimetric technique which provides an index of the degree of elevation of the gamma globulin fraction of the serum of patients irrespective of their disease. Dilution of serum with solutions of the salts of heavy metals was found to precipitate various protein fractions according to the concentration of metal used. Fig. 1 illustrates the curve of protein precipitation from normal serum at various concentrations of $CuSO_4$. Electrophoretic analyses showed that at the lower concentrations the precipitate consisted almost entirely of gamma globulin. By means of this curve it was possible to estimate a concentration of $CuSO_4$ that caused minimal precipitation of protein from normal serum. When hepatitis serum showing slight elevation of the gamma globulin fraction was diluted with $CuSO_4$ solution of this concentration, precipitation occurred, and the amount of precipitate was proportional to the increase in gamma globulin. The precipitated globulin could be accurately determined by turbidity measurements because the protein precipitated as a diffuse finely particulate suspension.

Zn, Hg, Pb, Cd, and Ur salts were found to produce an effect similar to that of $CuSO_4$.

[1] Moore, D. B., Pierson, P. S., Hanger, F. M., and Moore, D. H., *J. Clin. Invest.*, 1945, **24**, 292.

[2] Kunkel, H. G., and Hoagland, C. L., *J. Clin. Invest.*, 1947, **26**, 1060.

[3] MacLagan, N. F., *Brit. J. Exp. Path.*, 1944, **25**, 234.

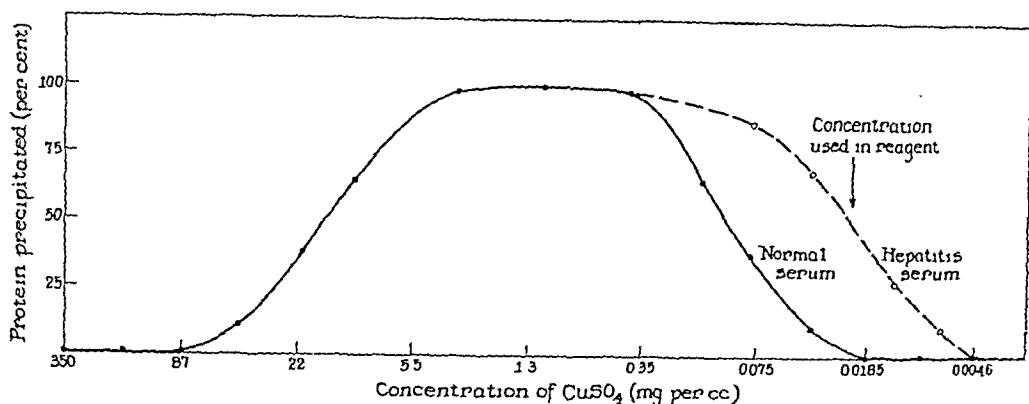[4] Wolff, E. K., *Tr. Roy. Soc. Trop. Med. and Hyg.*, 1939, **32**, 707.

FIG. 1.

Per cent of the total protein of normal and hepatitis serum precipitated following a 60-fold dilution with various concentrations of $CuSO_4 \cdot 5H_2O$ in distilled water.

Curves illustrating the effects of various concentrations on the precipitation of protein from serum closely resembled the curve for $CuSO_4$ in Fig. 1. When buffered solutions were used, $ZnSO_4$ was found to have certain advantages over $CuSO_4$.

The effect of the solutions of heavy metals in precipitating serum proteins was altered by small changes in pH and ionic strength. The results in Table I illustrate the comparative effect of the $CuSO_4$ solution usually used as reagent on normal and hepatitis serum under various conditions. The best differentiation between the two types of serum occurred at pH 6.5-7.5 in the presence of as low an ionic strength as it was practical to use.

Various concentrations of $CuSO_4$ reagent could be used for estimating changes in the globulin fraction. The concentration of 23 mg of $CuSO_4 \cdot 5H_2O$ per liter which causes minimal precipitation with normal serum was found to be the most useful. A zinc sulfate solution containing 24 mg of $ZnSO_4 \cdot 7H_2O$ per liter gives similar results, and has the advantage that it can be permanently buffered with barbiturate, whereas copper sulfate solution with barbiturate forms a precipitate. For routine use the solution eventually employed contained, per liter, 24 mg of $ZnSO_4 \cdot 7H_2O$, 280 mg of barbituric acid, and 210 mg of sodium barbiturate; the pH was 7.5.

Procedure. Measure one volume of serum (0.05 ml) into 60 volumes of either the $CuSO_4$ or the buffered $ZnSO_4$ reagent (3 ml). Allow to stand for 30 minutes, shake, and then read the turbidity in the spectrophotometer at 650 mμ.

The turbidity produced was translated into units by applying a standard curve similar to the one used for the thymol turbidity test.[5]* All values reported in this paper were determined by means of a Coleman Jr. spectrophotometer at 650 mμ. A colorimeter can also be used. In the absence of these instruments, a rapid estimate of the globulin elevation can be made by direct visual observation of the turbidity produced. The time necessary for flocculation to occur was a simple index of the globulin elevation. Some of the sera showing very high globulin levels demonstrated flocculation in a few minutes. The buffered $ZnSO_4$ reagent produced flocculation somewhat more rapidly than did

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[5] Shank, R. E., and Hoagland, C. L., *J. Biol. Chem.*, 1946, **162**, 133.

* 3 cc of a $BaCl_2$ solution (containing 1.15 g $BaCl_2 \cdot 2H_2O$ per 100 cc) is made to 100 cc in a volumetric flask with 0.2N H_2SO_4. This $BaSO_4$ suspension gives a turbidity equivalent to 20 units. By assigning the value 20 units to the optical density reading obtained in a colorimeter or a spectrophotometer at 650 μ with the $BaSO_4$ suspension, a standard curve can be constructed by drawing a line through the point obtained in the above manner and the 0 point on ordinary graph paper. The type of cuvette is not important as long as the same type is used for constructing the standard curve as for routine readings.

TABLE I.

Turbidity Produced by Adding 3 cc of a 2.3 mg % Solution of $CuSO_4$ to 0.05 cc of Serum Under Different Conditions of pH and Ionic Strength. Turbidities Are Expressed in Units Described in Text.

	pH	3.0	3.5	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.2	7.5	8.0	8.5		Ionic strength
Turbidity																
Normal serum		0	0	0	2	4	13	19	14	9	6	2	0	0	}	.01
Hepatitis serum		0	2	3	4	6	19	31	31	31	24	19	0	0	}	
Normal serum		0	0	0	0	0	0	4	2	0	0	0	0	0	}	0.1
Hepatitis serum		0	0	0	0	0	4	9	6	5	3	0	0	0	}	

the $CuSO_4$ reagent. Normal serum did not flocculate for at least 12 hours with the $ZnSO_4$ reagent while serum from patients with very slight elevation of the gamma globulin fraction usually flocculated within 4 hours.

The protein precipitated in the reaction dissolved readily on dialysis against sodium cyanide and the latter could be removed by further dialysis against barbital buffer at pH 7.8. Electrophoretic analysis of the protein treated in this manner revealed that the chief component precipitating in the reaction was a gamma globulin plus small amounts of other fractions (Fig. 2). This was true of the precipitate from 4 different sera that were studied. It appeared as if small amounts of other protein fractions, which normally would not precipitate at such a concentration of metallic salt, were carried down with the gamma globulin of these abnormal sera. Numerous observations of electrophoretic patterns were carried out on whole sera from patients showing different values for the copper and zinc turbidity reactions. In every case the increase in the gamma globulin com-

ponent correlated well with the intensity of the reaction. Fig. 3 illustrates the close relationship between the turbidity as measured by dilution of serum with the zinc reagent and the gamma globulin concentration as calculated from electrophoretic patterns of various pathological sera showing approximately normal albumin levels.

Certain sera with elevated lipid levels showed a marked increase in the beta globulin fraction. This increase was not reflected in the copper turbidity reaction. It is well known that such beta globulin peaks will often disappear almost completely by extraction of the lipids from the serum[6] and do not represent a true picture of the protein migrating in this fraction.

Addition of albumin to a positively reacting serum decreases the amount of globulin precipitated. Fig 4 demonstrates the *in vitro* change in the turbidity produced with the copper reagent upon the addition of increas-

[6] Longsworth, L. G., and MacInnes, D. A., *J. Exp. Med.*, 1940, 71, 77.

FIG. 2.

Ascending and descending electrophoretic patterns of the protein precipitated in the copper turbidity reaction. The major peak has the mobility of gamma globulin.
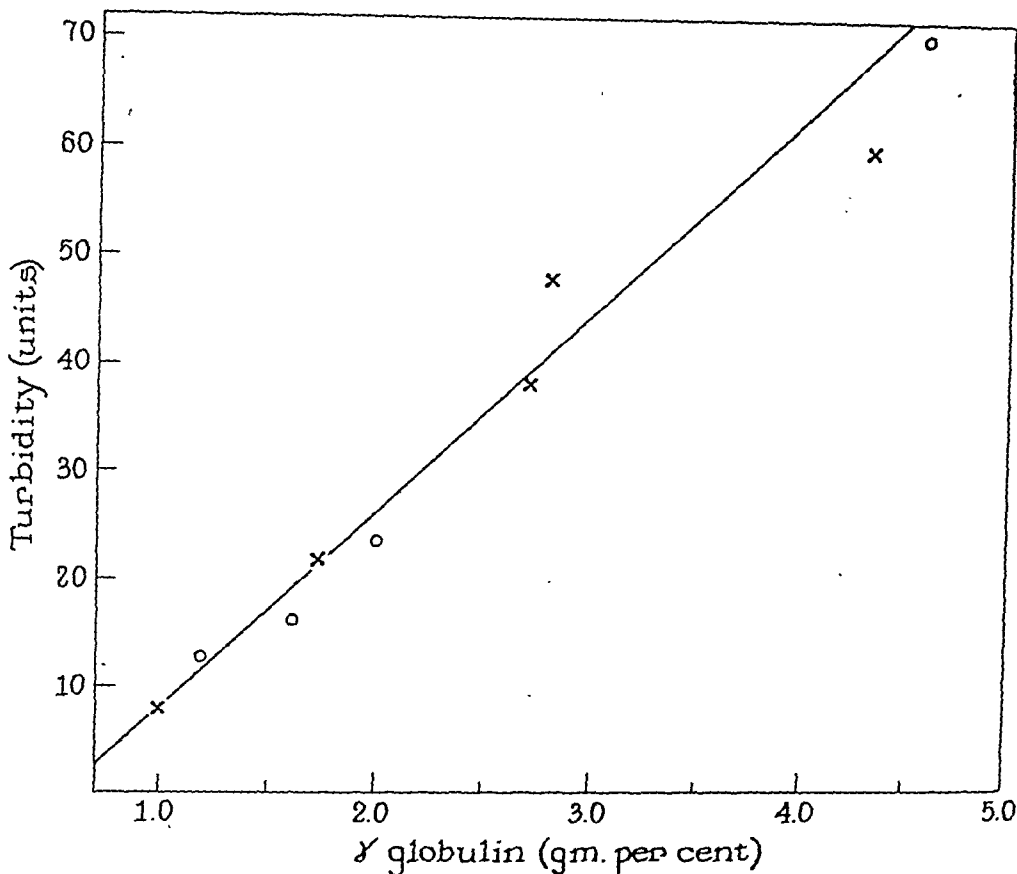
FIG. 3.

The correlation between the turbidity as measured in the zinc turbidity reaction and the gamma globulin concentration as calculated from electrophoretic patterns of various pathological sera. X = liver disease; O = other conditions.

ing amounts of human serum albumin. The intravenous administration of large amounts of concentrated human albumin to patients with liver disease demonstrated that a slight fall in the intensity of the reaction occurred and that it was similar to the fall expected from the *in vitro* experiments. The fact that an albumin deficit without hyperglobulinemia will not cause the copper turbidity reaction to become positive is clear, however, from observations on patients with nephrosis. The copper turbidity reaction was found normal despite the albumin deficit found in this disease.

Approximately 1000 determinations with the copper and zinc turbidity reactions have now been carried out on various sera. The normal range was found to lie between 2 and 8 units. Certain sera with globulin levels above 6 g % have shown values as high as 80 units. In these sera more than half of the protein was precipitated. *Fig. 5 illustrates the close relationship between the turbidity in units and the globulin level of the serum.* The reaction is not specific for liver disease but depends on the degree of elevation of gamma globulin. Serum from patients with multiple myeloma, for example, showed turbidity in proportion to the increase in gamma globulin. *Forty-one patients with cirrhosis of the liver were tested and all were found to demonstrate a positive reaction.* The test was of particular value when applied serially throughout the course of an acute illness. *Fig. 6 shows the markedly delayed elevation in gamma globulin as determined by the zinc turbidity test*
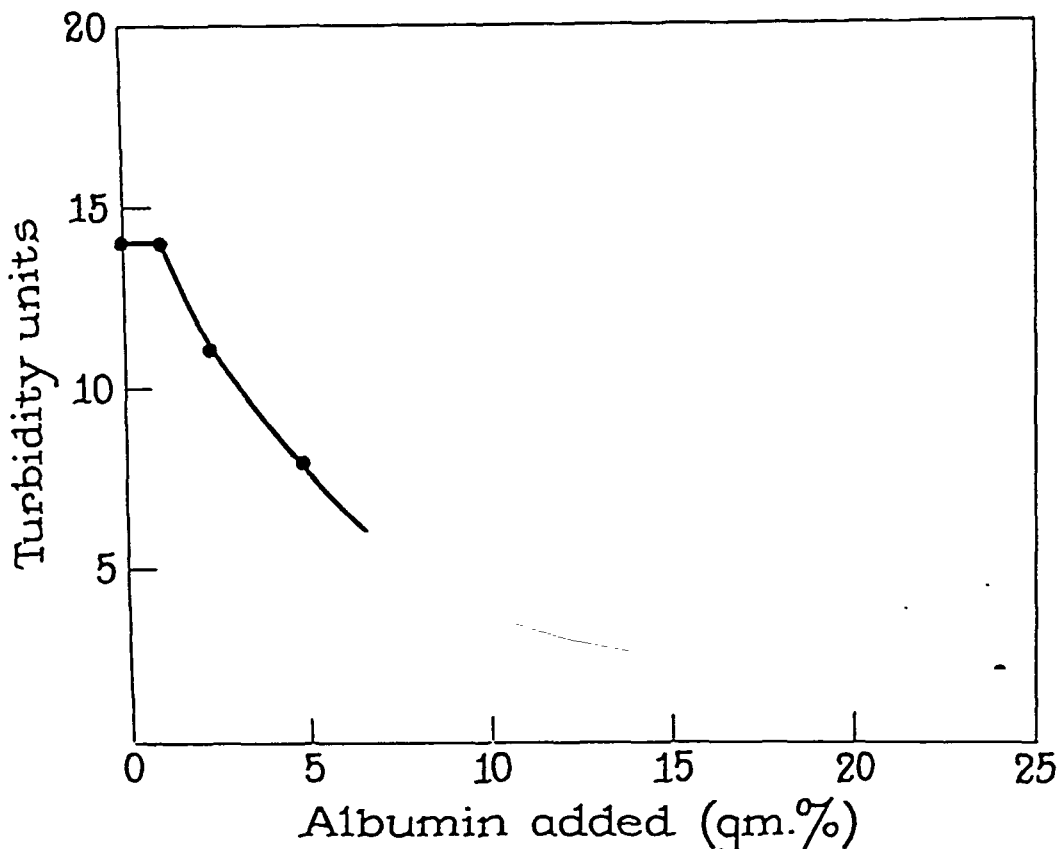
FIG. 4.

The effect of adding increasing amounts of concentrated human albumin to hepatitis serum on the intensity of the copper turbidity reaction.

during the course of a recurrence of acute infectious hepatitis. Similar changes with a delayed rise and prolonged elevation of the gamma globulin level have also been observed following an uncomplicated attack of acute infectious hepatitis.

Discussion. The Takata-Ara reaction and its many modifications, which have long been utilized for the diagnosis of liver disease, depend on the precipitation of protein from serum with a high globulin level. The mechanism of these reactions has always been obscure, but one of the substances added to serum in performing the tests is the metallic salt, $HgCl_2$. The study of the effect of various concentrations of heavy metals on the precipitation of protein from serum has furnished an understanding of this reaction. Curves similar to the ones illustrated in Fig. 1 for

$CuSO_4$ may be constructed for various mercury salts and concentrations which will precipitate globulins from abnormal sera may be obtained. The Takata-Ara reaction does not employ the dilution technique and is only positive when marked elevation of the globulin level occurs.

The concentrations of zinc and copper sulfates used in the reagents described above were useful for detecting elevations in gamma globulin in liver disease. However, other concentrations may also be used for different purposes. For example, a slightly higher concentration will precipitate globulin from serum with a normal or an abnormally low globulin level. The procedure of determining protein aberrations by the turbidity developed in dilute, metallic salt solutions is so simple in use and consistent in results that it may find
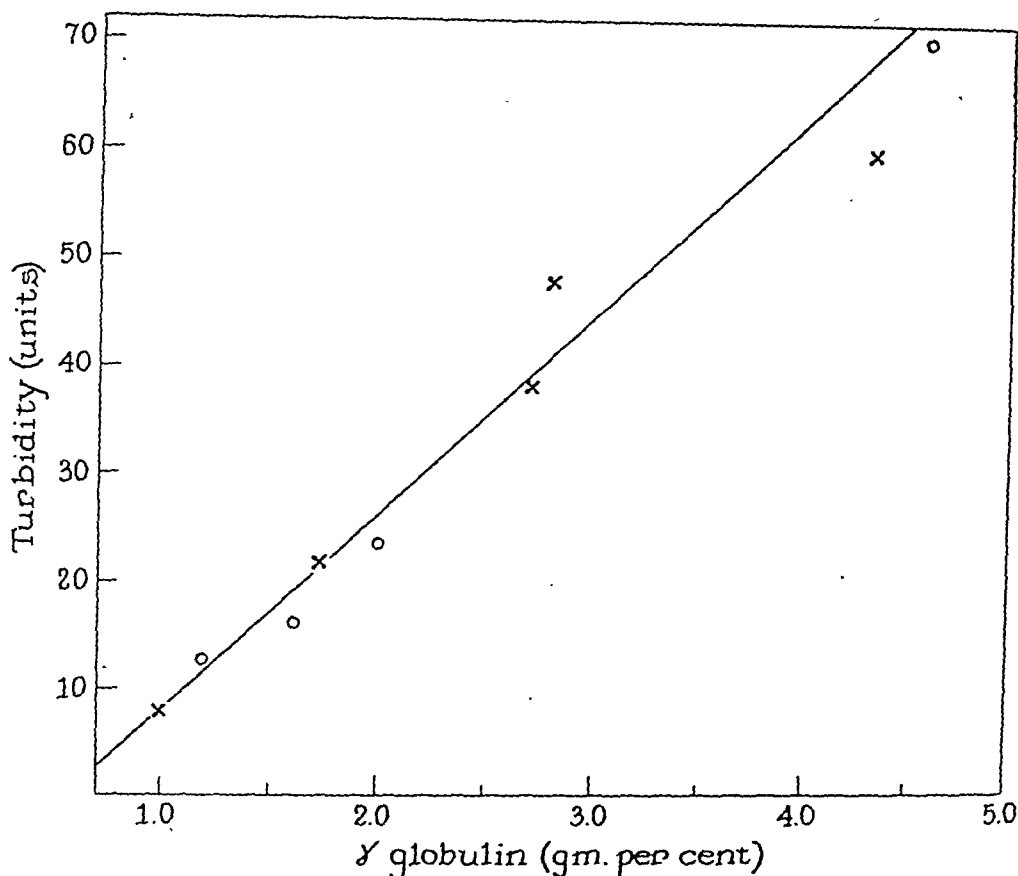
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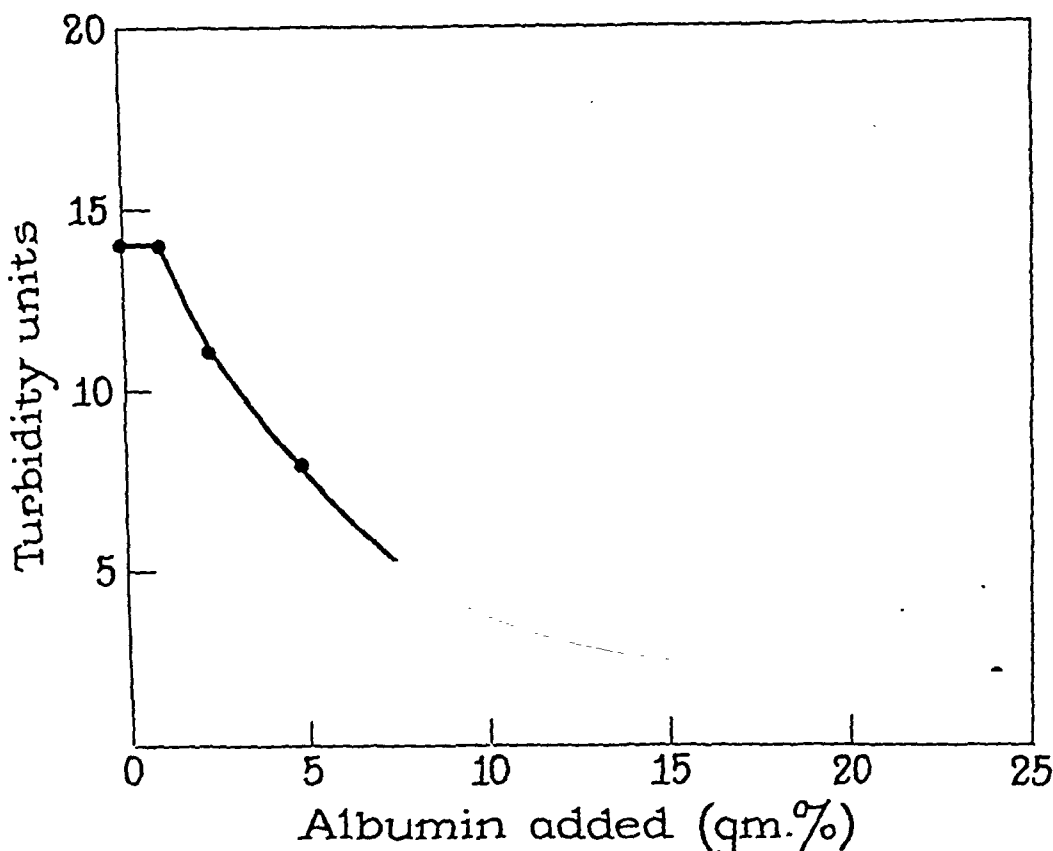
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Discussion. The Takata-Ara reaction and its many modifications, which have long been utilized for the diagnosis of liver disease, depend on the precipitation of protein from serum with a high globulin level. The mechanism of these reactions has always been obscure, but one of the substances added to serum in performing the tests is the metallic salt, $HgCl_2$. The study of the effect of various concentrations of heavy metals on the precipitation of protein from serum has furnished an understanding of this reaction. Curves similar to the ones illustrated in Fig. 1 for

$CuSO_4$ may be constructed for various mercury salts and concentrations which will precipitate globulins from abnormal sera may be obtained. The Takata-Ara reaction does not employ the dilution technique and is only positive when marked elevation of the globulin level occurs.

The concentrations of zinc and copper sulfates used in the reagents described above were useful for detecting elevations in gamma globulin in liver disease. However, other concentrations may also be used for different purposes. For example, a slightly higher concentration will precipitate globulin from serum with a normal or an abnormally low globulin level. The procedure of determining protein aberrations by the turbidity developed in dilute, metallic salt solutions is so simple in use and consistent in results that it may find
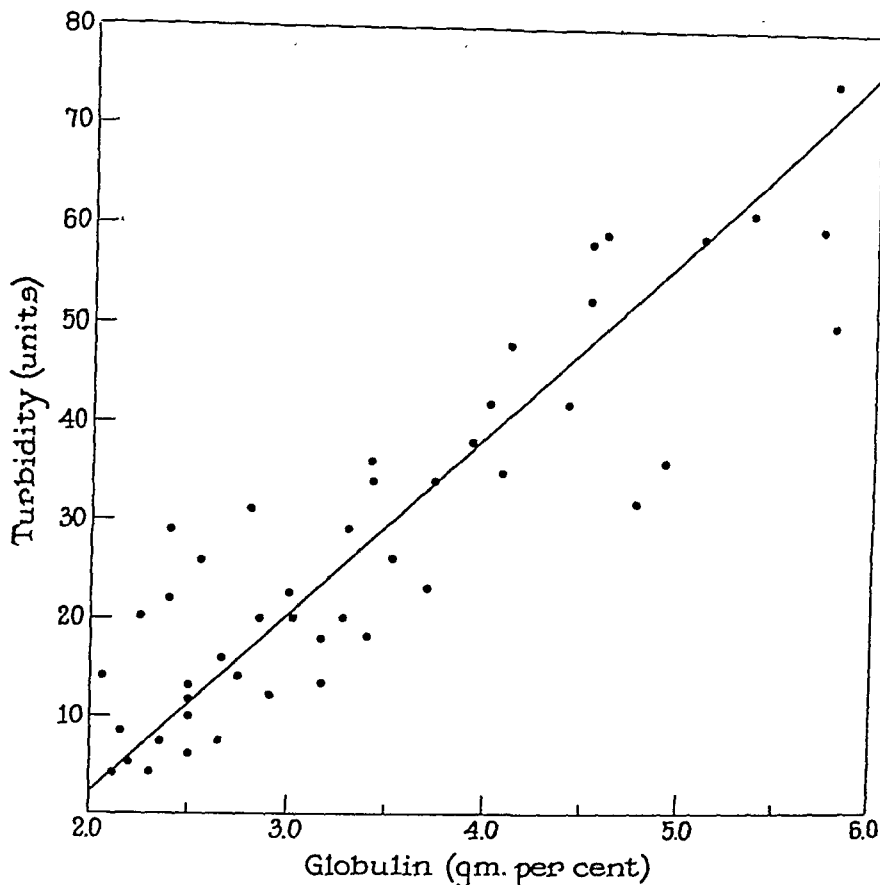
FIG. 5.

The correlation between the turbidity as measured in the zinc turbidity test and the total globulin level as determined by the Howe fractionation in various pathological sera.

application for a wide variety of purposes.

Measurable increases in the total globulin of serum in liver disease and in other conditions are almost always due to elevation of the gamma globulin fraction.[2,7] The beta globulins may show significant changes electrophoretically but these depend mainly on the lipid level of the serum and do not represent true protein alterations. As a result, any technique which measures increases in gamma globulin in pathological sera is really a measure of the elevation in total globulin.

Significant curves of the globulin changes during the course of acute infectious hepatitis

were not obtained until the copper and zinc turbidity techniques were adopted. The delayed rise and prolonged elevation during the course of this disease is of considerable interest. It suggests the possibility that the globulin elevation may reflect the production of antibodies rather than a disturbance in liver function. The albumin level of the serum was often unaltered during the period of hyperglobulinemia. Serial determinations of the thymol turbidity test closely paralleled those of the globulins during the convalescent phase of infectious hepatitis, but marked differences were readily apparent in cases of cirrhosis of the liver. In this condition the thymol turbidity reaction was of considerably less value and showed little relation to the globu-

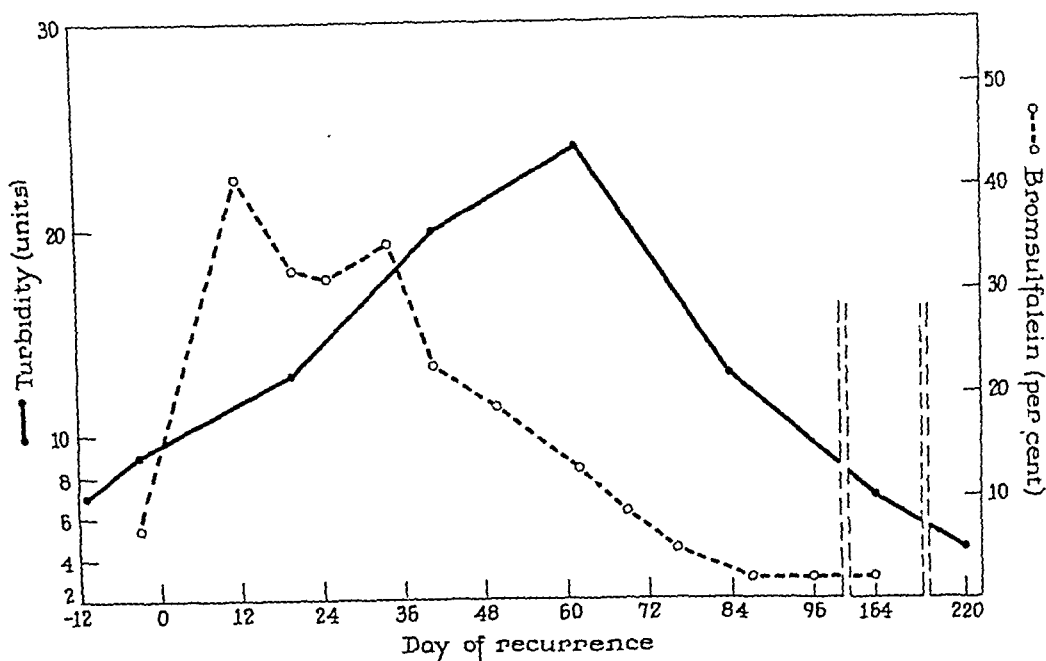7 Gray, S. J., and Guzman Barron, E. S., J. Clin. Invest., 1943, 22, 191.

FIG. 6.
The delayed rise and prolonged elevation of the gamma globulin level in the serum following a recurrence of infectious hepatitis as determined by the zinc turbidity test. The curve for bromsulfalein retention is added for purposes of comparison.

lin aberration.[8]

In chronic infectious hepatitis alterations in the globulin components of the serum may be the only indication of persisting liver disease. In previous studies[8,9] it was demonstrated that the thymol turbidity test was positive more often than any other single liver function test that could be applied to patients with persistent vague complaints more than 6 months after an attack of acute infectious hepatitis. Electrophoretic patterns showed that values for the thymol test parallelled the changes in the gamma globulin fraction closely in these patients. All patients with abnormalities in the thymol test also showed abnormalities in the copper and zinc turbidity reactions. In addition, the latter frequently remained positive after the thymol test had become negative. The zinc turbidity test, although not specific for liver disease, was found to be the most sensitive method of

detecting a lingering hepatitis. Five patients have been observed who developed cirrhosis of the liver following infectious hepatitis. A characteristic feature of this group was marked elevation of the gamma globulin fraction of the serum electrophoretically. In two of these patients the gamma globulin reached values above 6 g %. These aberrations were easily detected by the addition of the metal reagents to serum. The use of this test provided a rapid method of screening patients with persistent symptoms following infectious hepatitis in searching for the more severe complications of the disease.

The main advantage in the use of a test of this type instead of the cephalin flocculation or the thymol turbidity reaction is that a single known alteration in the serum is measured. This alteration is an elevation in the gamma globulin fraction. The other reactions depend partly on this alteration but also on other factors less well understood, such as the lipid level of the serum.

Another application of the zinc turbidity test has been in the study of the gamma globu-

[8] Kunkel, H. G., and Hoagland, C. L., *Am. J. Med.*, in press.

[9] Kunkel, H. G., and Hoagland, C. L., Proc. Soc. Exp. Biol. and Med., 1946, 62, 258.

lin changes associated with the development of antibodies following scarlet fever in patients with normal convalescence and patients developing rheumatic fever. Increased turbidity was found to closely parallel the rise in antistreptolysin and antistreptokinase titers. These results will be reported in a separate communication.

Summary. 1. When serum with abnormally high gamma globulin concentration is diluted with a solution containing a certain small amount of copper or zinc sulfate, a turbid precipitate forms and the optical density of the suspension is proportional to the concentration of gamma globulin.

2. Such an estimation of increase in gamma globulin really measures the total globulin elevation in pathological sera because hyperglobulinemia is almost always due to an alteration in the gamma globulin fraction of the serum.

3. The test has proved useful for determining alterations in gamma globulin during the course of an acute illness such as infectious hepatitis.

4. It was found to be of particular value in detecting persistent liver disease following infectious hepatitis. In a group of 41 patients with cirrhosis of the liver the reaction was positive in every case.

16044 P

Black Pigmentation in Feathers of Buff Orpington Chicks Caused by Vitamin D Deficiency.*

ANNABELLE DECKER AND JAMES MCGINNIS. (Introduced by A. R. Kemmerer.)
From the Department of Poultry Husbandry, State College of Washington, Pullman, Wash.

Abnormal amounts of black pigment in feathers of New Hampshire chicks fed vitamin D deficient diets were observed by Glazener, Mattingly, and Briggs.[1] Domm[2] reported that feeding desiccated thyroid caused abnormal black coloring in Brown Leghorn males, females, and capons. Juhn and Barnes,[3] working with capons of the same breed, produced similar results. Both the New Hampshires and the Brown Leghorns normally have some black pigment in the feathers. Buff Orpingtons, on the other hand, do not normally have black pigment in the feathers. It was of interest, therefore, to determine whether a deficiency of vitamin D would cause the black-pigmented feathers in this breed.

Experimental Methods. The percentage composition of the vitamin D deficient basal diet fed in this experiment is as follows: ground yellow corn 30.0, ground wheat 25.5, ground barley 10.0, dehydrated alfalfa 5.0, B-Y riboflavin concentrate (250 μg riboflavin/g) 0.5, ground limestone 2.0, dicalcium phosphate 1.5, soybean oil meal (expeller) 20.5, fish meal 4.0, salt (iodized) 0.5, and soybean oil 0.5. Manganese was added at a level of 75 p.p.m.

Chicks were examined and weighed at weekly intervals. At 6 weeks the feather color of each chick was given a numerical score, ranging from 0 to 5 depending upon the intensity of black pigment in the back feathers. A score of 0 indicated no black pigment whereas a score of 5 indicated very markedly blackened feathers. Since some of the chicks were extremely slow feathering, it was not possible to score all of them.

At the end of the 6-week period, a few chicks from each vitamin D deficient diet

1 Glazener, E. W., Mattingly, J. P., and Briggs, G. M., *Poultry Sci.*, 1946, 25, 85.

2 Domm, L. V., *Anat. Rec.*, 1929, 44, 227.

3 Juhn, M., and Barnes, B. O., *Am. J. Physiol.*, 1931, 98, 463.

TABLE I.
Effect of Vitamin D Deficiency on Feather Pigmentation in Buff Orpington Chicks.

Supplement to basal diet	Avg wt at 6 wk g		No. of chicks scored	Feather color			
				% showing black		Color score	
None	312	304*	12	100	100*	3.1	2.9*
	296		13	100		2.6	
Vit. D†	482	470	13	0	0	0	0
	458		11	0		0	
Iodinated casein‡	318	309	11	100	100	2.0	2.7
	295		12	100		3.3	
Iodinated casein + vit. D	422	471	11	0	0	0	0
	520		9	0		0	

* Average of duplicate groups.
† 100 A.O.A.C. units/100 g diet.
‡ 15.0 g/100 lb diet.

were placed on a ration containing adequate vitamin D, and were observed from day to day for several weeks.
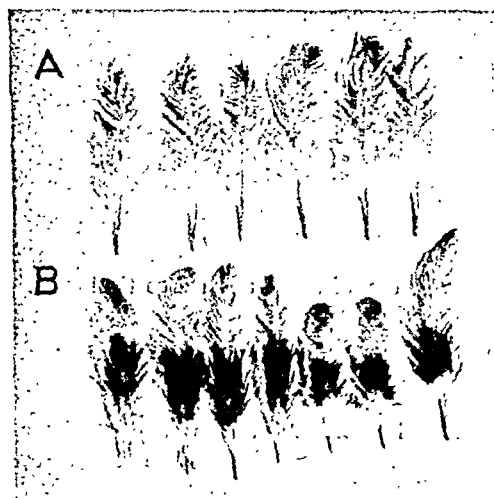

FIG. 1.
Black banded feathers of Buff Orpington chicks fed vitamin D-deficient diets. (A) Normal feathers taken from birds receiving adequate amounts of vitamin D. (B) Abnormal feathers from birds receiving vitamin D-deficient diets. Note banding effect caused by growth of new feathers of normal color after vitamin D supplementation.

Results. The results are presented in Table I and Fig. 1. At 4 weeks of age most of the chicks fed on the vitamin D deficient diets showed varying amounts of gray to black color. At 6 weeks of age intense black was noticeable in the feathers of the humeral tract of the vitamin D deficient birds. The under-color of the back, breast, and leg feathers as well as color at the base of the flight feathers and wing coverts, was black. All of the chicks fed the vitamin D deficient diets grew some feathers with black pigment. The degree and distribution of black pigmentation, however, varied considerably. Without exception, all of the chicks receiving the diets supplemented with vitamin D or vitamin D plus iodinated casein had normal buff colored feathers, free of black pigment. Under the conditions of this experiment, thyroactive iodinated casein did not increase the deposition of black pigment in chicks fed a vitamin D deficient diet or a diet supplemented with this vitamin.

At the end of the experiment, several chicks from each lot were placed on a chick ration containing vitamin D. The immature black feathers of chicks fed the vitamin D deficient diets during the experimental period grew

with a normal buff color at the base following the change in diets. This growth of normal colored feathers was noted at about five days after the diets were changed. Fig. 1 shows the banding effect produced in the feathers.

Summary. In an experiment with Buff Orpington chicks, a breed normally having no black pigment in the feathers, it was found that a deficiency of vitamin D caused a widespread deposition of black pigment in the feathers. This abnormal blackening was prevented by supplementing the diet with vitamin D.

The feeding of thyroactive iodinated casein failed to increase the deposition of black pigment in chicks fed either a vitamin D deficient or vitamin D supplemental diet.

16045

Response of Spontaneous Lymphoid Leukemias in Mice to Injection of Adrenal Cortical Extracts.*†

L. W. LAW AND ROBERT SPEIRS. (Introduced by C. C. Little.)

From the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, and the Department of Zoology, University of Wisconsin, Madison.

Striking alterations in blood elements have been observed to result from injections of pituitary adrenotropic hormone in various species of animals.[1,2] The blood picture observed within a few hours after injection is as follows: decrease in leukocyte count, an absolute lymphopenia and a corresponding absolute polymorphonuclear leukocytosis. Statistically significant decreases in the weights of lymphoid tissues, excepting the spleen, following injections of adrenotropic hormone, indicate a profound influence on normal maintenance of lymphoid tissue by this hormone.[3] Histological studies indicate specific degenerative changes of normal lymphocytes in all lymphatic structures with repair and recovery after definite time intervals.[4] These changes are mediated through the adrenal cortex and similar alterations, although at possibly different time intervals, occur following injections of adrenal cortical extracts.[2]

In view of these profound effects on normal lymphocytic tissues, we have undertaken a study of the effects of adrenal cortical extract on the immature elements in spontaneous lymphoid leukemias in mice. Earlier studies have indicated some relationship between the adrenals and the growth of transmitted animal leukemias. Sturm and Murphy[5] showed that adrenalectomy reduced the natural resistance of rats to a transplantable lymphoid leukemia. The rate of growth of the leukemia was also apparently increased following adrenalectomy. It has been also[6] shown that desoxycorticosterone acetate and two adrenal cortical extracts decreased the susceptibility of rats to a transplanted leukemia. Complete disappear-

1 Dougherty, T. F., and White, A., *Science*, 1943, 98, 367.

2 Dougherty, T. F., and White, A., *Endocrinology*, 1944, 35, 1.

3 Dougherty, T. F., and White, A., PROC. SOC. EXP. BIOL. AND MED., 1943, 53, 132.

4 Dougherty, T. F., and White, A., *Am. J. Anat.*, 1945, 77, 81.

5 Sturm, E., and Murphy, J. B., *Cancer Research*, 1944, 4, 384.

6 Murphy, J. B., and Sturm, E., *Science*, 1944, 99, 303.

ance of a transplantable lymphoid tumor in mice followed by recurrence but definite retardation in growth after administration of 11-dehydro-17 hydroxycorticosterone (Compound E) has been reported.[7]

In the C58 strain of mice approximately 90% of mice of both sexes develop leukemia, the majority of which are lymphoid in origin. Early symptoms of leukemia can be detected within the strain by periodic palpation of lymph nodes. Usually a single node is initially involved[8] followed by a progressive systemic course of the disease. An incidence of approximately 80% leukemia, involving a significantly greater number of females than males, has been observed in the inbred RIL strain. The majority of these leukemias involve initially and principally the thymus. The first symptom observed is usually that of dyspnea and the animals are at this time in the terminal stage of the disease.

The leukocyte count is definitely elevated in leukemias of both strains of mice, although not severely, and immature lymphoid forms have been found in the peripheral blood of all leukemias observed within these strains. There occurs a progressive leukocytosis and a definite increase in circulating immature forms as the disease runs its course.

In the following experiment we are reporting 13 cases of spontaneous lymphoid leukemia arising in the inbred leukemic strains of mice, C58 and RIL (including 2 hybrids between these strains) which were injected with adrenal cortical extracts. Most of these leukemias were in the terminal stages of the disease (from 5 to 16 days following discovery of symptoms) when administration of adrenal cortical extract was begun. Mice of the C58 strain had moderate to severe lymphadenopathy of the axillary, inguinal and cervical lymph-nodes, moderate to severe splenomegaly and numerous immature leukocytes in the peripheral circulation. Mice of the RIL strain showed dyspnea and thoracic

enlargement and in some cases had subcutaneous lymph-node and splenic involvement. In 5 of the 13 cases of spontaneous leukemia, biopsy tissue (axillary or inguinal lymph node) was taken prior to treatment, part of which was saved for histological section and the other part inoculated into mice of the strain of origin of the leukemia. This was done to confirm further the diagnosis of leukemia.

Leukemic mice were inoculated initially, either intraperitoneally or subcutaneously with from 0.1 to 0.5 cc of adrenal cortex extract (aqueous) or lipoadrenal cortex.[‡] Since the lipoadrenal cortical extract gave the most favorable effects, subsequent inoculations were continued with this at usually 0.1 cc inoculation every 24 hours. Several animals received this dose every 12 hours for a considerable period of time and optimum response as determined by blood analyses was obtained. Blood analyses were made at 0, 3, 6, 9, 24, 72, and 168 hours and at various times thereafter throughout the life of the animal.

The blood picture obtained is characterized by: (a) decrease in total white blood-cell count, (b) decrease in absolute number of lymphocytes with a corresponding polymorphonuclear leukocytosis and decrease in absolute number of immature forms (lymphocytes and lymphoblasts). Following initial administration of adrenal cortical extract, the maximum blood alterations were noted at 6 hours. After continued hormone administration these effects were enhanced so that in the majority of mice given daily injections for 168 hours or longer there resulted a severe lymphopenia and decrease in absolute number of circulating immature blood cells. Two cases, however, both in the C58 strain proved refractory after 72 hours and showed a progressive increase in circulating leukocytes and immature forms. Recovery to a leukemic blood picture tended to occur after 9 hours following injection of adrenal cortical extract. However, after continued daily injections over a relatively long period of time an effect of the extract has been observed in animals 24 hours after injection

[7] Heilman, F. R., and Kendall, E. C., *Endocrinology*, 1944, 34, 416.

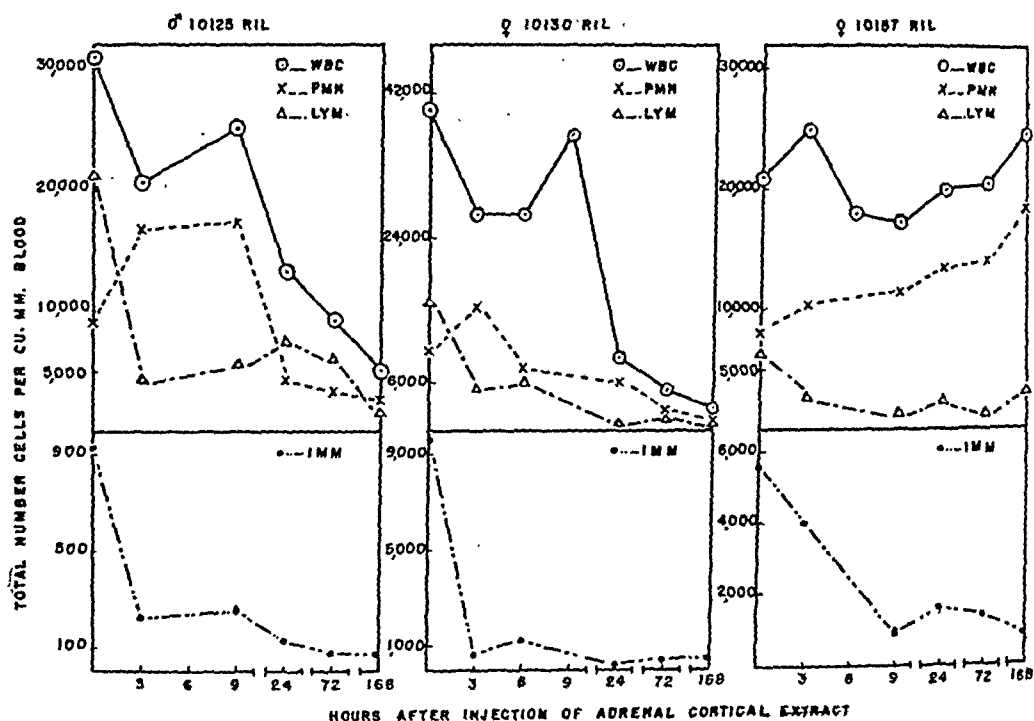[8] Law, L. W., *Proc. Nat. Acad. Sci.*, 1947, 33, 204.

FIG. 1.

Effect of injections of adrenal cortical extract on the white blood cell counts of three mice of the RIL strain with lymphoid leukemia. Injections were given every 12 hours and blood analyses were made usually at 6 hours following injection. Blood analyses for these mice were continued throughout life and are explained further in the text.

(Fig. 1 and 2).

Degenerating lymphocytes both mature and immature were observed in peripheral blood 6 hours after initial injection of adrenal cortical hormone and constituted as much as 20% of the differential count in several leukemic animals which had received injections over a period of 10 days to 2 weeks or longer. Nuclear degenerative changes were prominent in these cells. Dougherty and White[2] have not described a peripheral removal of lymphocytes in their experimental observations in normal mice but suggest this as a possible mechanism in normal lymphocyte dissolution. Whether the phenomenon observed here is characteristic of the lymphocytes of leukemic mice or is related to a strain difference in the mice employed must await further experimentation.

Favorable effects of adrenal cortical extract on hemoglobin levels in leukemic mice have not been observed, although it is possible that the decline in hemoglobin levels is not as precipitous in treated as in control leukemic animals.

Profound palliative effects were noted within 24 to 48 hours in the majority of leukemic mice. These effects were: complete disappearance of the symptoms of dyspnea and thoracic enlargement and marked regression in infiltrated inguinal, axillary, cervical and mesenteric lymph-nodes. Definite regression of the spleen was noted in some leukemic animals but this was not constant throughout the treated series. It has been reported in normal mice receiving injections of adrenotropic hormone that the spleen did not show the characteristic weight decrease observed for other lymphoid tissues.[3] The characteristic response to adrenal cortical extract in a typical case, ♀ 10130 RIL, was as follows: Sixteen days after appearance of symptoms of leukemia this animal was in the terminal stages of leukemia. There was extreme dyspnea, moderate involvement of all subcutaneous lymph nodes, each measuring approximately 3 x 3

**HOURS AFTER INJECTION OF ADRENAL
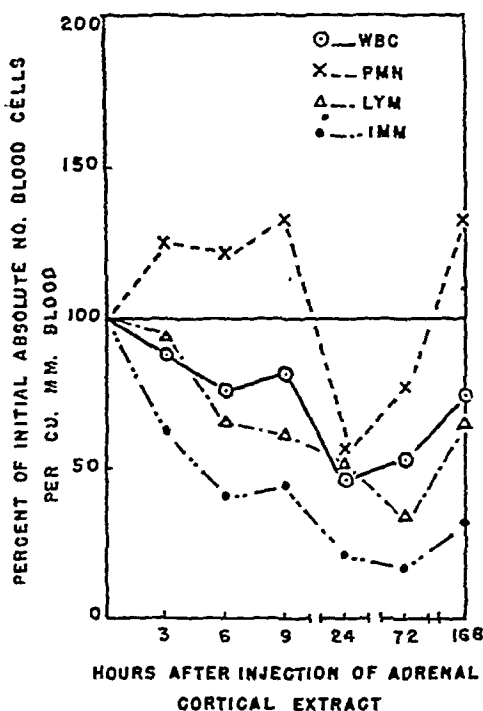CORTICAL EXTRACT**

FIG. 2.

Mean percentage increase or decrease of initial
number of blood cells in leukemic mice receiving
daily injections of adrenal cortical extract. Each
point on curve represents the mean of data obtained
from at least 10 mice. Mean percentage increase at
168 hours is the result of including data of 2 mice
which became refractory at this time.

mm. a moderately involved spleen and palpable mesenteric nodes. Biopsy tissue from an inguinal lymph node showed severe infiltration of immature forms and mice of the RIL strain inoculated with the remaining tissue of the inguinal node developed leukemia within 3 to 4 weeks. The leukocyte count was elevated to 40,500 and 24% of the circulating cells were lymphoblasts. Lipoadrenal cortex was injected subcutaneously 0.1 cc twice daily. Within 48 hours all subcutaneous nodes had completely regressed, there was no evidence of dyspnea and the spleen and mesenteric nodes showed definite regression. This animal continued to show regression with daily inoculation of extract and died 30 days after appearance of symptoms at which time the subcutaneous lymph nodes were normal in size, the thymus and spleen showed slight

to moderate infiltration and the liver and kidneys were greatly infiltrated. (See Fig. 1 for blood response in this animal.)

Lymph nodes, spleen, thymuses, liver and other organs were removed following death of the animal for histological study. In addition several inguinal nodes were removed at 2 and 3 days following initial injection. Severe alterations in infiltrated lymph-nodes and thymuses were observed in leukemic mice which had received numerous injections of adrenal cortical extract. Extensive degenerative changes of the immature lymphocytes were evident. Nuclei were pyknotic and of various sizes and bizarre shapes. Hyperplasia of the cytoplasm was marked in numerous immature forms. Nuclear debris was scattered throughout the organ with very little evidence of phagocytosis. There was a severe depletion of immature forms and a "washed-out" appearance of nodes and thymuses. Recovery and repair processes were not noticeable. Mitotic figures were not observed. In contrast, the alterations in the spleen of leukemic mice receiving long continued injections were more localized. Numerous macrophages were present, filled with nuclear debris and there were many areas of histiocytic infiltration wherein the histiocyte-like cells had abundant cytoplasm but did not exhibit phagocytosis.

In inguinal lymph nodes examined at 2 and 3 days there were in evidence areas of recovery with many macrophages present, filled with nuclear debris. In addition in scattered areas there were present large reticular cells lying free in the sinuses but not actively phagocytic.

Degenerative changes of the immature lymphocytes were not found in the non-hemopoietic tissues studied.

Definite weight decreases of spleen. thymus and subcutaneous lymph-nodes of treated leukemic mice were obtained. The effect on thymic mass was most severe resulting in a mean weight of 270 $\pm$ 90 mg in the experimental series compared with 663 $\pm$ 66.5 mg in leukemic controls.

From the small number of mice observed in this preliminary series, it is impossible to state the effect on life expectancy. Two leukemic mice of the RIL strain have lived 30

and 42 days respectively with continued daily intraperitoneal inoculations. We have observed the course of the disease in more than 50 leukemic animals of this strain and none has survived for this period of time.

Summary. Injections of adrenal cortical extract into 13 mice of the inbred C58 and RIL strains in the terminal stages of spontaneous lymphoid leukemia resulted in the following responses: (a) An acute response (maximum at 6 hours following initial injection) resulting in a decrease of circulating leukocytes of the blood, a lymphopenia with a corresponding absolute polymorphonuclear leukocytosis and a decrease in the number of circulating immature lymphocytes. (b) These blood alterations become more pronounced following continuous daily injections and tend not to return to the leukemic blood picture within 24 hours after injection of the extract. (c) Regression of infiltrated thymuses and subcutaneous lymph nodes was observed. Regression of the spleen, although definite, was not so pronounced as that observed in other lymphoid tissues. (d) Extensive degenerative changes in immature lymphocytes in thymuses and lymph nodes resulting in pyknosis, dissolution and depletion of these cells. In the spleen similar changes occurred but were not so generalized. Pronounced degenerative changes in immature lymphocytes was observed in thymuses and lymph nodes of mice receiving daily injections over a relatively long period of time. Recovery and repair in these lymphoid organs was slight.

16046

Intertransformability of *Salmonella simsbury* and *Salmonella senftenberg*.[*]

P. R. Edwards, Alice B. Moran, and D. W. Bruner.

From the Department of Animal Pathology, Kentucky Agricultural Experiment Station, Lexington, Ky.

It is recognized[1-7] that profound changes may be induced in H antigens of *Salmonella* by cultivating the organisms in serums containing appropriate H agglutinins. Such

1 Kauffmann, F., Z. Hyg., 1936, **119**, 104.

2 Gnosspelius, A., Z. Hyg., 1939, **121**, 528.

3 Kauffmann, F., and Tesdal, M., Z. Hyg., 1937, **120**, 168.

4 Edwards, P. R., and Bruner, D. W., J. Bact., 1939, **38**, 63.

5 Bruner, D. W., and Edwards, P. R., J. Bact., 1941, **42**, 467.

6 Edwards, P. R., and Bruner, D. W., J. Bact., 1942, **44**, 289.

7 Edwards, P. R., and Moran, A. B., Proc. Soc. Exp. Biol. and Med., 1946, **61**, 242.

changes seem to occur particularly in monophasic cultures. Thus, in the stools of a person infected with *S. cholerae-suis* var. *kunzendorf* (VI,VII:1,5) Kristensen and Bojlen[8] found an organism with the biochemical properties of Kunzendorf whose antigens were VI,VII:c. Such variants are easily produced *in vitro* by cultivating Kunzendorf cultures in 1,5 serum. The writers have encountered 12 of these variants occurring naturally among cultures from man and swine. Likewise, Edwards and Moran[7] found that monophasic strains of *S. minnesota* (XXI,XXVI:b) were easily changed to a form with new H antigens (XXI,XXVI:z_{33}) by cultivation in b serum. Cultures with the latter formula occurred naturally among strains isolated from sewage by A. A. Hajna and it seems very

8 Kristensen, M., and Bojlen, K., Zbt. f. Bakt., I, Orig., 1936, **136**, 295.

likely that they arose from XXI,XXVI:b cultures by induced variation which occurred in nature.

Observations like the above cast suspicion on *Salmonella* types with unusual H antigens.

A type with hitherto unrecognized H antigens may be a derivative of some known type whose antigens have been changed. Bruner and Edwards[9] described *S. simsbury* (I,III,-XIX:z_{27}) as a new type with distinct H antigens. Subsequently 10 additional representatives of *S. simsbury* were recognized among cultures received for typing. While the organism seemed to constitute a valid *Salmonella* type and was so accepted, the observations on *S. minnesota* indicated the need for further study of the relationship of *S. simsbury* to *S. senftenberg* (I,III,XIX:g,s,t).

Nine cultures of *S. simsbury* were plated and single colonies selected. The single colony isolations were examined and found to have O and H antigens characteristic of the type. They were inoculated into semisolid agar to which had been added z_{27} serum freed of O agglutinins by absorption with boiled cultures of *S. senftenberg*. One culture gave evidence of change in the H antigens by spreading slightly in the first transfer in serum-semisolid medium. From this spreading growth a form indistinguishable from *S. senftenberg* was isolated. Similar changes were observed in the remaining 8 strains of *S. simsbury* after 6 to 11 transfers in the medium.

The cultures of *S. simsbury* which had been changed to I,III,XIX:g,s,t and 10 typical cultures of *S. senftenberg* were then planted in semisolid medium containing g,s,t serum. These cultures proved more resistant to change than did the original *S. simsbury* strains. After 10 to 12 serial transfers over a period of 2 months they began to spread very slowly through the medium. After several additional transfers a form which had H antigens unlike any known *Salmonella* type was isolated from all the cultures. To this form the symbol z_{34} was applied.

The z_{34} forms were then transferred serially in semisolid medium which contained both g,s,t and z_{34} serums. After numerous transfers over a period of 3 months one of the *S. senftenberg* cultures spread rapidly through the medium. From this culture a form having the antigens I,III,XIX:z_{27} was isolated. The remaining z_{34} cultures gave no evidence of change.

The agglutinative characteristics of the various forms are given in Table I. Absorption tests confirmed the results obtained by agglutination. The g,s,t form of *S. simsbury* removed all agglutinins from *S. senftenberg* serum. Likewise, the z_{27} form of *S. senftenberg* exhausted *S. simsbury* serum. The changes brought about may be summarized as follows:

I,III,XIX:z_{27} ⟶ I,III,XIX:g,s,t
I,III,XIX:g,s,t ⟶ I,III,XIXz_{34} ⟶
    I,III,XIX:z_{27}
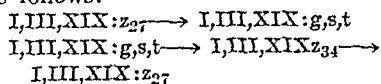
S. simsbury and *S. senftenberg* must have descended from the same ancestral stock. Possibly this ancestor was a typical diphasic *Salmonella* which shifted normally between g,s,t and z_{27} phases. A stock culture of *S. simsbury* that had been transferred regularly on agar slants for 5 years was found to possess H antigens like those of *S. senftenberg*. The conditions under which the culture was kept made it highly improbable that it was mislabelled. This apparent spontaneous change of a stock culture from z_{27} to g,s,t supports the hypothesis that the ancestor of the two types was diphasic. A second possibility is that the parent culture was a more complex monophasic strain which spontaneously divided into two simpler components. Loss variation of this sort occurs in *S. hormaechei* (XXIX,Vi:z_{30},z_{31}) which produces XXIX,Vi:z_{30} and XXIX,Vi:z_{31} variants which are quite stable but which can be changed each into the other by growth in appropriate serums (Edwards[10]). A third possibility is that the very rare *S. simsbury* is an induced variant of the commonly occurring *S. senftenberg* just as XXI,XXVI:z_{33} seems to be an induced variant of XXI,-XXVI:b. The difficulty of the g,s,t ⟶ z_{27} variation does not support this view.

It is not possible to state exactly how

9 Bruner, D. W., and Edwards, P. R., Proc. Soc. Exp. Biol. and Med., 1942, **50**, 174.

10 Edwards, P. R., *J. Bact.*, 1946, **51**, 523.

and 42 days respectively with continued daily intraperitoneal inoculations. We have observed the course of the disease in more than 50 leukemic animals of this strain and none has survived for this period of time.

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16046

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P. R. EDWARDS, ALICE B. MORAN, AND D. W. BRUNER.

From the Department of Animal Pathology, Kentucky Agricultural Experiment Station, Lexington, Ky.

It is recognized[1-7] that profound changes may be induced in H antigens of *Salmonella* by cultivating the organisms in serums containing appropriate H agglutinins. Such changes seem to occur particularly in monophasic cultures. Thus, in the stools of a person infected with *S. cholerae-suis* var. *kunzendorf* (VI,VII:1,5) Kristensen and Bojlen[8] found an organism with the biochemical properties of Kunzendorf whose antigens were VI,VII:c. Such variants are easily produced *in vitro* by cultivating Kunzendorf cultures in 1,5 serum. The writers have encountered 12 of these variants occurring naturally among cultures from man and swine. Likewise, Edwards and Moran[7] found that monophasic strains of *S. minnesota* (XXI,XXVI:b) were easily changed to a form with new H antigens (XXI,XXVI:z_{33}) by cultivation in b serum. Cultures with the latter formula occurred naturally among strains isolated from sewage by A. A. Hajna and it seems very

1 Kauffmann, F., *Z. Hyg.*, 1936, **119**, 104.

2 Gnosspelius, A., *Z. Hyg.*, 1939, **121**, 528.

3 Kauffmann, F., and Tesdal, M., *Z. Hyg.*, 1937, **120**, 168.

4 Edwards, P. R., and Bruner, D. W., *J. Bact.*, 1939, **38**, 63.

5 Bruner, D. W., and Edwards, P. R., *J. Bact.*, 1941, **42**, 467.

6 Edwards, P. R., and Bruner, D. W., *J. Bact.*, 1942, **44**, 289.

7 Edwards, P. R., and Moran, A. B., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 242.

8 Kristensen, M., and Bojlen, K., *Zbt. f. Bakt.*, I, Orig., 1936, **136**, 295.

16047

Hemoprotein from Root Nodules and Nitrogen Fixation by *Rhizobium.*[*]

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Although the necessity of root nodule bacteria for fixation of molecular nitrogen by leguminous plants has been established since 1886, experimental proof that these organisms are the responsible agent still is lacking. In spite of numerous claims of fixation by pure cultures of the bacteria, few such claims withstand critical examination.[1] The identification of the red pigment in root nodules as a hemoprotein[2-5] led inevitably to the suggestion it was directly concerned with the fixation reaction. Among the support for this proposal was an experiment by Virtanen and Laine[6] in which addition of nodular extracts to pure cultures of *Rhizobium* apparently enabled them to fix appreciable quantities of nitrogen. The fixation was particularly striking if oxalacetic acid was added with the extract. This claim appeared surprising in view of the difficulty in inducing excised nodules to assimilate molecular nitrogen although they still contain both bacteria and pigment. Nevertheless, because of its obvious importance, extensive tests of the possibility were undertaken. Such tests are limited to the summer months when sufficient nodules can be grown to provide pigment for extensive replication. During the past 2 summers we

have made these tests but have obtained no evidence that the presence of the pigment or any other constituent of the extract of nodules from soybean or pea stimulate the free living *Rhizobium* to fix nitrogen. Since these experiments were completed, a new report from Virtanen[7] states that more extensive trials fail to confirm their first experiment; we shall summarize here only the experimental variations tested and the results.

Materials and Methods. Nodules were taken from leguminous plants grown on a nitrogen-poor sand in a cold-frame. Twice during the growing season the plants were watered with Hoagland's N-free nutrient solution. The nodules were picked into cold water, drained, and macerated with an equal quantity of water. The extract was pressed through cheese-cloth, centrifuged for 20 minutes, then passed through a Berkefeld *N* filter and suitable aliquots added to 6 oz prescription bottles containing 25 ml N-free medium. This medium was: Allison's salt mixture, 1.5 g; sucrose, 10 g; water, 1000 ml. Traces of Fe and Mo were supplied together with 1 ml/liter yeast water (2 mg N) for growth factors. When the soybean organism was used, 1 ml of a mesquite gum hydrolysate was also added. Various-species of *Rhizobium* were grown on agar slants (Medium 79 of Fred and Waksman), and aliquots of their suspensions added to the N-free medium. The incubation was at 30°C for 7 days; total nitrogen was determined by a semimicro Kjeldahl procedure sensitive to 0.02 mg.

Experimental variations tested in attempts to secure fixation included:

1. *Source of nodules.* Soybeans inoculated with *Rhizobium japonicum* 534 and Canada field peas with *R. leguminosarum* 317 were used. Nodules from plants 6 to 9 weeks old

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1 Wilson, P. W., *The Biochemistry of Symbiotic Nitrogen Fixation,* University of Wisconsin Press, 1940.

2 Kubo, H., *Acta Phytochim.* (Japan), 1939, **11**, 195.

3 Burris, R. H., and Haas, E., *J. Biol. Chem.,* 1944, **155**, 227.

4 Keilin, D., and Wang, Y. L., *Nature,* 1945, **155**, 227.

5 Virtanen, A. I., *Nature,* 1945, **155**, 747.

6 Virtanen, A. I., and Laine, T., *Suomen Kemistilehti,* 1945, **18B**, 39.

7 Virtanen, A. I., Jorma, J., Linkola, H., and Linnasalma, A., *Acta Chemica Scand.,* 1947, **1**, 90.

TABLE I.
Agglutination and Absorption Tests.

Serums	Senftenberg (g,s,t)	Senftenberg (z_{34})	Senftenberg (z_{27})	Simsbury (z_{27})	Simsbury (g,s,t)	Simsbury (z_{31})
Senftenberg (g,s,t)						
Unabsorbed	20000	0	500	500	20000	0
Absorbed by						
Simsbury (g,s,t)	0	0	0	0	0	0
Senftenberg (z_{34})	10000	0	500	500	10000	0
Senftenberg (z_{27})	10000	0	0	0	10000	0
Simsbury (z_{27})						
Unabsorbed	0	0	10000	10000	0	0
Absorbed by						
Senftenberg (z_{27})	0	0	0	0	0	0
Simsbury (z_{34})	0	0	10000	10000	0	0
Simsbury (g,s,t)	0	0	10000	10000	0	0
Senftenberg (z_{34})						
Unabsorbed	0	5000	500	500	0	2000
Absorbed by						
Senftenberg (g,s,t)	0	2000	500	500	0	2000
Simsbury (z_{34})	0	200	0	0	0	0
Simsbury (z_{34})						
Unabsorbed	0	5000	500	500	0	5000
Absorbed by						
Simsbury (z_{27})	0	5000	0	0	0	5000
Senftenberg (z_{34})	0	0	0	0	0	200

Figures indicate highest dilutions at which agglutination occurred.
0 indicates no agglutination at 1 to 200.

S. simsbury arose but since it obviously came from the same ancestor as *S. senftenberg*, it seems logical to omit *S. simsbury* from the Kauffmann-White classification and to assign *S. senftenberg* the formula I,III,XIX:-g,s,t—z_{27}.

Summary. By growth in serums it was possible to change *S. simsbury* (I,III,XIX:z_{27}) into *S. senftenberg* (I,III,XIX:g,s,t) and vice-versa. The z_{27}———→g,s,t change was accomplished without difficulty but the reverse was done only by first transforming the H antigens to a hitherto unrecognized form (z_{34}) and then changing them to z_{27}. It is recommended that *S. simsbury* be dropped from the classification and that the formula of *S. senftenberg* be written I,III,XIX:g,s,t—z_{27}.

by the summary in Table I no such gains were obtained. It is concluded that if fixation occurs it is too weak to be detected by the Kjeldahl method. The possibility that the slight gains occasionally observed possess significance is now being tested using the much more sensitive isotopic method.

Summary. Fixation of molecular nitrogen could not be induced in free-living cultures of *Rhizobium* by supplying them with extracts of root nodules containing the hemoglobin-like pigment. Nine experiments were made during 2 growing seasons. Variations in technique included: species of bacteria; source of nodular extract; addition of oxalacetic, α-ketoglutaric and citric acids; and method of preparing the extract. Analyses of the results showed that gains of 0.2-0.3 mg N were required for statistical significance, but even these slight increases were not consistently obtained.

16048

Effect of Induced Liver Cirrhosis on the Reproductive System of the Male Rat.[*]

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While it has been shown repeatedly that the physiology of reproduction is dependent upon the maintenance of a normal pituitary-gonadal balance, it is also becoming increasingly evident that intact liver function is essential to the normal metabolism of the sex steroid hormones. The results of *in vitro* experiments performed by numerous investigators have provided ample evidence that the liver is the principal organ responsible for the inactivation and removal from the blood of the natural estrogen of the body (Silberstein et al.,[1] Zondek,[2] Engel and Navratel,[3] Heller et al.,[4] Heller,[5] and Engel,[6]). Inactivation of endogenous estrogens by the liver has been reported by numerous workers

using varying techniques. Talbot[7] induced liver damage in female rats with carbon tetrachloride in alcohol and found an increase in uterine weight of 200% by the third day of the experiment. From this he concluded that the poisoned animals were exposed to an increased concentration of blood estrogen because of the impaired inactivating capacity of the liver.

Other experiments (Golden and Severinghaus,[8] Biskind and Mark,[9] Biskind,[10] Krichesky, Benjamin and Slater,[11]) were devised to divert the gonadal hormones directly into the portal or into the systemic circulation. When the portal route was used the hormones were promptly inactivated, as shown by failure to maintain the sex accessories of castrate animals, whereas the hormones escaped inactivation when they drained directly into

1 Silberstein, F., Engel, P., and Molnar, K., *Klin. Wchnshr.*, 1933, 12, 1693.

2 Zondek, B., *Skand. Arch. fur Physiol.*, 1934, 70, 133.

3 Engel, P., and Navratel, E., *Biochem. Z.*, 1937, 202, 434.

4 Heller, C. G., Heller, E. J., and Severinghaus, E. L., *Am. J. Physiol.*, 1939, 126, 530.

5 Heller, C. G., *Endocrin.*, 1940, 26, 619.

6 Engel, P., *Endocrin.*, 1941, 29, 290.

7 Talbot, N. B., *Endocrin.*, 1941, 25, 290.

8 Golden, J. B., and Severinghaus, E. L., PROC. SOC. EXP. BIOL. AND MED., 1938, 39, 361.

9 Biskind, G. R., and Mark, J., *Bull. Johns Hopkins Hosp.*, 1939, 65, 212.

10 Biskind, G. R., PROC. SOC. EXP. BIOL. AND MED., 1941, 47, 266.

11 Krichesky, B., Benjamin, J. A., and Slater, C., *Endocrin.*, 1943, 32, 345.

TABLE I.
Summary of Nitrogen Fixation Experiments.

Treatment	Exp. I		Exp. II		Exp. III	
	No. of samples	Mean N, mg/25 ml	No. of samples	Mean N, mg/25 ml	No. of samples	Mean N, mg/25 ml
Control	2	2.95	2	2.57	2	2.65
None	2	2.96	3	2.62	3	2.63
α-Ketoglutarate	2	2.96	4	2.51	4	2.72
Oxalacetate	2	2.99	3	2.60	4	2.64
Citrate	2	3.03	4	2.66	3	2.73
	Exp. IV		Exp. V		Exp. VI	
Control	3	3.25	2	1.63	2	3.38
None	4	3.24	—	—		
α-Ketoglutarate	3	3.25	2	1.66	2	3.27
Oxalacetate	2	6.37*	2	1.38	3	3.73
Citrate	3	3.19	2	1.69	2	3.25
	4	6.38*				
	Exp. VII		Exp. VIII		Exp. IX	
Control	2	2.41	2	3.20	2	2.64
None	2	2.49	2	3.19	3	2.91
α-Ketoglutarate	4	2.43	3	3.36	2	2.99
Oxalacetate	3	2.38	3	3.22	—	—
Citrate	2	2.45	3	6.14*	2	2.94

Extract of nodules containing pigment added to all samples; those marked with asterisk given twice the level of others. Controls kept at 3°C, others at 30°C.

gave the most satisfactory extracts.

2. *Method of Extraction.* In about one-half the experiments, the nodules were extracted *in the cold under carbon monoxide.* Before its addition to the medium the pigment was reoxygenated for 20 minutes.

3. *Filtration.* The finest filter tried was a Seitz, but a Berkefeld N was usually used as it did not clog. *Extracts of nodules from plants in the flowering stage were so viscous that they passed only through the cheesecloth.*

4. *Organism.* Species of *Rhizobium* tested, singly or in mixtures were *R. japonicum*, *R. leguminosarum*, *R. melilotii*, and *R. trifolii.*

5. *Additions.* Various organic acids, oxalacetic, α-ketoglutaric and citric, often implicated in fixation schemes were added (15 mg/25 ml).

6. *Incubation.* In some experiments the bacterial cells were incubated for 3 days in the N-free medium before the addition of the extract as was suggested by Virtanen and Laine;[6] in others, the cells and extract were added at the same time.

7. *Miscellaneous.* Two to 4 replicates were made of each treatment. The controls were treated in the same way as the experimental

flasks but were kept at 3°C. The quantity of pigment added varied with the preparation, but an attempt was made to standardize this so that the extract added to 25 ml contained 2 to 3 mg N. In 2 experiments twice the usual level of extract was supplied. About 0.1-0.2 mg N was contained in the bacteria used as inoculum.

Results. The agreement in nitrogen content among replicates is illustrated by the data from a typical experiment: Control, 2.60, 2.53; plus extract, 2.62, 2.61, 2.63; plus extract and oxalacetate, 2.58, 2.58, 2.62; plus extract and α-ketoglutarate, 2.66, 2.50, 2.63, 2.62; plus extract and citrate, 2.55, 2.72, 2.58, 2.69 mg per 25 ml. Statistical analysis of the data from 9 experiments (113 samples) indicated that the standard deviation of the means of duplicates was about 0.15 mg and of the means of quadruplicates about 0.10 mg. Therefore, the means of the replicates for each treatment would have to exceed that of the control by 0.2 to 0.3 mg even to approach statistical significance. Actually, of course, one would wish greater differences for so important a conclusion, *e.g.*, a gain of at least 0.5 to 1 mg of N per bottle. As can be seen

TABLE I.
Effect of Carbon Tetrachloride Intubation of the Weight of the Sex Accessories in the Male Rat
(0.05 cc CCl₄ in 50% Alcohol Administered 3 × Weekly).

Group No.	No. of animals	Duration of treatment, days	Avg body wt, g	Avg sex acces. wt, mg	Avg testis wt, mg
1	8	0	163	845	1841
2	6	22	160	637	1921
3	7	43	154	390	1663
4*	7	60-80	155	278	1840
5*	4	88+40	207	476	1586

* In Group 5 carbon tetrachloride was administered for 88 days. The animals were then maintained for 40 days without treatment before they were sacrificed.

fur was rough; the backs were arched and they lost weight rapidly. Most of these were sacrificed in order to obtain fresh tissues for histologic study. The mortality among the other 2 groups was almost the same (approximately 20%) and only few of the surviving animals showed observable evidence of illness. Liver necrosis appeared in the animals receiving 0.025 cc of carbon tetrachloride by the 19th day and by the 11th day in the 0.05 cc group. Cirrhosis was present in the 0.025 cc group by the 58th day and by the 44th day in 0.05 cc group. The latter dose, therefore, produced maximum hepatic injury in the shortest time with no greater mortality than in the .025 cc group.

The liver manifested a series of progressive architectural changes not unlike that seen in human cirrhosis, that is, cloudy swelling and fatty infiltration in early stages, followed by central necrosis of the lobule with attempts at healing in the longer term experiments. Fibrosis, as well as gross nodularity was evident after 44 days of carbon tetrachloride feeding. In animals with more advanced liver failure, ascites was usually present.

From the onset of carbon tetrachloride intubation the kidneys of all animals became pale in color and later took on a yellowish cast. In the long term experiments the color was usually a chocolate brown. The histologic picture was remarkably constant with an early cloudy or albuminoid swelling followed by more severe swelling of the cells and accompanied by a coarsening of the reticulum and a clouding or granulation of the cytoplasm. This persisted throughout the experiment and was nearly constant at all dosage levels.

In the second experiment the pertinent data are given in Table I. The average weight of the sex accessories (including seminal vesicles, coagulating gland, ampullary gland, and the lateral, dorsal and ventral lobes of the prostate) was 845 mg in the untreated animals. After 22 days of treatment with 0.05 cc carbon tetrachloride the average weight of the accessories was 637 mg or approximately a 25% decrease. After 43 days of treatment the average weight was decreased by 53% to 390 mg. The group receiving treatment for 60 to 80 days showed accessories weighing only 278 mg, a 67% decrease below the untreated controls. The final group treated for 88 days and then maintained for 40 days without treatment before being sacrificed showed partial recovery in the weight of the accessories. In this group the accessories averaged 476 mg, a decrease of 44% below the controls and an increase of 70% over the group treated for 60 to 80 days and sacrificed immediately thereafter.

Although there was striking atrophy of the sex accessories following liver damage by carbon tetrachloride, yet the results indicate that the testicles were not so greatly affected. There was great variation in testicular weight among animals of all groups and because the number of animals used was small the data given in Table I are not considered significant.

Histologic examination of the testes revealed no striking architectural disorganization of the seminiferous tubules. Spermatogenesis was not greatly impaired in the majority of animals. In only 2 of 32 testes examined was marked testicular damage observed. This was indicated by absence of mature sperm and marked atrophy of spermatogenic cells.

the systemic circulation and consequently the sex accessories were maintained. The usual method was to transplant the gonad into the mesentery to facilitate drainage into the portal vein or to transplant the gonad into the axilla or body wall to prevent portal drainage. Steroid hormone pellet implants in the spleen of castrate rats was the method favored by Biskind and his associates.

Further indication of hepatic inactivation is provided by the study of steroid hormone metabolism in the presence of experimental or clinical liver damage. Extensive liver disease seems to impair hormone inactivation so that estrogenic effects are usually intensified Pincus and Martin[12] report an 80% increase in estrogen effectiveness in rats with experimental liver damage. Human observations (Edmondson et al.,[13] Glass, et al.,[14,15] Gilder and Hoagland,[16] and others) imply that acute or chronic liver disease, if extensive enough, may be associated with impairment of steroid hormone metabolism. Such disease may be followed by impairment of testicular function or the development of the full blown syndrome of testicular atrophy, gynecomastia and torso alopecia. The latter syndrome has been ascribed by one of us (Glass) to the effects of circulating biologically active estrogens emanating from failure of hepatic inactivation. To test this thesis it was deemed advisable to study the sex organs of male rats with experimental liver damage. The spontaneous effects of liver damage on the sex organs of the experimental animal have not been adequately investigated.

Method. Eighty-four mature male Wistar albino rats weighing from 150 to 210 g were used and were maintained on Rockland rat pellets and lettuce. All animals were intubated thrice weekly and given various amounts of

12 Pincus, G., and Martin, D. W., *Endocrin.*, 1940, **27**, 838.

13 Edmondson, H. A., Glass, S. J., and Soll, S. N., Proc. Soc. Exp. Biol. and Med., 1939, **42**, 97.

14 Glass, S. J., Edmondson, H. A., and Soll, S. N., *Endocrin.*, 1940, **27**, 749.

15 Glass, S. J., Edmondson, H. A., and Soll, S. N., *J. Clin. Endocrin.*, 1944, **4**, 54.

16 Gilder, H., and Hoagland, C. L., Proc. Soc. Exp. Biol. and Med., 1946, **61**, 62.

carbon tetrachloride in 50% alcohol. Two experiments were carried out.

Experiment I. A pilot experiment using 35 animals was undertaken to determine the amount of carbon tetrachloride that would produce maximum hepatic damage with minimum mortality. The animals were divided into 3 approximately equal groups: Group A received 0.025 cc carbon tetrachloride in alcohol in doses of 0.5 cc; Group B received 0.05 cc in doses of 0.5 cc; Group C received 0.10 cc in doses of 1.0 cc. Animals from each group were sacrificed at selected intervals. Biopsies were carried out during the course of the treatment which continued for as long as 12 weeks in some animals. Macroscopic examination of the liver was made at autopsy and portions of the liver and kidney were prepared for histologic study.

Experiment II. Having determined an adequate dosage of carbon tetrachloride, a second experiment was carried out to determine the effects of hepatic injury on the male reproductive system. Forty-nine animals were used, 17 either died or were sacrificed for histologic material during the course of the experiment and the remaining 32 provided the quantitative data. The experimental animals were intubated 3 times per week with 0.05 cc carbon tetrachloride in 50% alcohol solution in doses of 0.5 cc. Five groups were employed: Group 1, animals serving as controls without treatment; Group 2, animals intubated over a period of 22 days; Group 3, animals intubated over a period of 43 days; Group 4, animals intubated over 60 to 80 days; Group 5, animals intubated for 88 days and then maintained without treatment for 40 days.

All animals were weighed before and after the experiment. The sex accessories were carefully dissected free of surrounding connective tissues and were weighed on a torsion balance. The testes were also removed and weighed. These structures and the liver and kidneys were preserved for histologic study.

Results. In the first experiment the mortality rate was greatest in those animals receiving the largest dose of carbon tetrachloride. Within a few days they appeared sickly; the

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In the second experiment the pertinent data are given in Table I. The average weight of the sex accessories (including seminal vesicles, coagulating gland, ampullary gland, and the lateral, dorsal and ventral lobes of the prostate) was 845 mg in the untreated animals. After 22 days of treatment with 0.05 cc carbon tetrachloride the average weight of the accessories was 637 mg or approximately a 25% decrease. After 43 days of treatment the average weight was decreased by 53% to 390 mg. The group receiving treatment for 60 to 80 days showed accessories weighing only 278 mg, a 67% decrease below the untreated controls. The final group treated for 88 days and then maintained for 40 days without treatment before being sacrificed showed partial recovery in the weight of the accessories. In this group the accessories averaged 476 mg, a decrease of 44% below the controls and an increase of 70% over the group treated for 60 to 80 days and sacrificed immediately thereafter.

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Discussion. The data presented indicate that the oral administration of carbon tetrachloride produces hepatic damage and results in atrophy of the secondary sex organs of the male rat as indicated by reduction of weight part of which may be accounted for by the atrophy of bled tissue and part by possible reduction in the amount of the secretions of these organs. This treatment causes no significant weight changes in the testes. Healing of the liver damage promptly follows withdrawal of the poisoning agent and is accompanied by partial recovery of the weight of sex accessory organs 40 days after cessation of carbon tetrachloride administration.

In explaining these data two possible hypotheses suggest themselves; (1) that hepatic damage and sex accessory atrophy may be produced by the toxicity of the carbon tetrachloride and (2) that liver damage due to carbon tetrachloride may cause failure of hepatic inactivation of endogenous estrogens resulting in an increased level of circulating estrogens.

In the first instance, it must be admitted that direct toxic effects of carbon tetrachloride on the accessories have not been eliminated in these experiments. The atrophy of the secondary sex organs may well be due to a direct toxic action of the poison in a manner similar to the damage produced in the liver. However, it would be remarkable that such toxicity would affect only the accessory organs and not the testes, especially since the latter have been reported to be highly sensitive to noxious agents. It is surprising that only 2 animals of 32 examined show degenerative changes in the testes and no significant weight loss. The accessory organs, on the other hand, in all animals, exhibit a weight loss ranging from 25 to 67% below untreated animals. If this atrophy is due to a direct toxicity of carbon tetrachloride, then these data suggest that in the doses employed, the poison affects the sex accessory organs preferentially and has little or no effect on the testes. A difference in threshold of these organs obtains some support from the findings of Simpson and Evans[17] that the sex accessories are less re-

sistant to androgen deprivation than the testicular tubules.

It should be pointed out also that in some unpublished experiments carried out in this laboratory in which hepatic damage was induced in rats by a low protein diet (4% casein with vitamin supplements), atrophy of the prostate and seminal vesicles was observed in the males. These results, without the use of a toxic agent, were qualitatively similar to those after carbon tetrachloride feeding. It should be recognized, however, that deficiency diets, by interference with normal metabolism, may serve as toxic agents in themselves.

A second possible explanation, that liver damage induced by carbon tetrachloride feeding may prevent hepatic inactivation of endogenous estrogens resulting in an increased level of circulating estrogens, may account adequately for the results reported here.[†] A high level of circulating estrogens, especially in the "free" form as suggested by Glass, Edmundson and Soll may induce secondary sex atrophy either by a direct inhibition of these organs or by inhibition of pituitary gonadotropin secretion. This view is supported by the findings of Morrione[18] that doses of estrogens insufficient to produce testicular damage in normal rats resulted in severe testicular damage when given to animals with livers damaged by carbon tetrachloride feeding. He suggested that these low doses were effective only in the latter group of animals (with damaged livers) because circulating estrogen levels in them were increased.

Healing of liver injury and recovery of the sex accessories following withdrawal of carbon tetrachloride feeding may be explained by either hypothesis. Withdrawal of the poison

17 Simpson, M. E., Li, C. H., and Evans, H. M., *Endocrin.*, 1944, **35**, 96.

† That circulating endogenous estrogens after hepatic injury are increased is supported by unpublished data from this laboratory in which bioassay showed approximately a thousand-fold increase in urinary estrogens excreted by female guinea pigs with carbon tetrachloride-damaged livers over that excreted by normal animals.

18 Morrione, T. G., *Arch. Path.*, 1944, **37**, 39.

permits healing and recovery by removing the deleterious agent or by healing of the liver and a return to normal steroid metabolism with consequent inactivation of endogenous estrogens.

Summary. Pathologic changes in the liver and kidneys of male rats fed carbon tetra-chloride is described. Striking atrophy of the secondary sex organs but no significant changes in weight of testes follows variable periods of carbon tetrachloride feeding. Withdrawal of the poisonous agent results in healing of the liver and partial weight recovery of the sex accessories.

16049 P

Catheterization of the Coronary Sinus and the Middle Cardiac Vein in Man.*

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A technique of intravenous catheterization of the coronary sinus has been developed recently in intact dogs by Goodale and Lubin,[1] using the Cournand intravenous catheter.[2] This method has been employed by Eckenhoff and co-workers[3] to measure coronary blood flow, using the nitrous oxide method developed by Kety and Schmidt for the determination of cerebral blood flow.[4] The procedure appears to be less hazardous than the method in intact dogs previously reported by Harrison and co-workers,[5] which involved the use of a brass balloon cannula. In a combined study of 20 different dogs,[1,3] as many as 7 catheterizations and 4 duplicate measure-ments of coronary flow have been performed on the same dog at monthly intervals. These developments suggested that a similar procedure might be followed in the determination of coronary blood flow in man. This paper is a preliminary report of 9 successful catheterizations of the coronary vessels in man.

Methods. Most of the patients studied had congenital heart disease. Consequently, catheterization of the heart of these individuals was primarily undertaken to obtain information concerning the nature of the cardiac anomalies.[6,7,8] Only one subject, (No. 7, Table I), had a normal heart. For catheterization of the right heart the technique of Cournand[2] was followed, using standard No. 7 catheters. Passage of the catheter into the coronary sinus or the middle cardiac vein was verified by (a) fluoroscopic control, (b) recording of pressures, and (c) determinations of blood oxygen and carbon dioxide contents. It was assumed that the coronary sinus had been successfully intubated if the systolic pressures were below ventricular and above auricular systolic pres-

1 Goodale, W. T., Lubin, M., and Banfield, W. G., in press.

2 Cournand, A., *Fed. Proc.*, 1945, 4, 207.

3 Eckenhoff, J. E., Hafkenschiel, J. H., Harmel, M., Goodale, W. T., Lubin, M., and Kety, S. S., in press.

4 Kety, S. S., and Schmidt, C. F., *Am. J. Physiol.*, 1945, 143, 53.

5 Harrison, T. R., Friedman, B., and Resnick, H., Jr., *Arch. Int. Med.*, 1936, 57, 927.

6 Bing, R. J., Vandam, L. D., and Gray, F. D., Jr., *Bull. Johns Hopkins Hosp.*, 1947, 80, 107.

7 Bing, R. J., Vandam, L. D., and Gray, F. D., Jr., *Bull. Johns Hopkins Hosp.*, 1947, 80, 121.

8 Bing, R. J., Vandam, L. D., and Gray, F. D., Jr., *Bull. Johns Hopkins Hosp.*, 1947, 80, 323.

TABLE I.
Blood Gas Values Obtained from Catheterization of Heart and Coronary Sinus.

No.	Date	Age, yrs	Sex	O_2 content R.A. vol. %	O_2 content R.V. vol. %	O_2 content C.S. vol. %	O_2 content F.A. vol. %	A-V oxygen difference Coronary F.A.-C.S.	Systemic F.A.-Rt. Heart
1.	5/31/46	16	M	18.8	18.5	6.7	22.9	16.2	4.4
2.	7/ 2	16	M	17.0	18.0	8.8	22.0	13.2	5.0
3.	26	17	F	18.1	17.8	8.3	24.6	16.3	6.8
4.	31	16	M	24.0	24.9	9.5	28.2	18.7	4.2
5.	8/ 5	14	F	15.2	17.3	6.3	21.2	14.9	5.0
6.	2/14/47	19	F	9.5	12.6	5.5	16.8	11.3	7.3
7.	5/ 2	23	F	—	13.4	5.0	16.2	11.2	2.8
8.	20	11	F	14.9	14.8	5.2	17.6	12.4	2.8
9.	23	28	F	18.3	19.9	8.9	24.9	16.0	6.6

R.A.—Right auricle.   C.S.—Coronary sinus.
R.V.—Right ventricle.   F.A.—Femoral artery.

sures,[9] and if the oxygen contents of the sinus blood were significantly below those of returning mixed venous blood.[1] Under fluoroscopy the catheter enters the coronary sinus just below the region of the tricuspid valve, slightly medial, superior and anterior to the inferior vena cava. When the catheter is in the coronary sinus, it is seen curved upward toward the base of the heart. When the middle coronary vein is intubated, the catheter lies alongside the lower borders of the heart with the tip directed toward the apex. In the first five cases intubation of these vessels was fortuitous. A similar experience has been reported by others.[10] In the remaining 4 cases, catheterization of the sinus was carried out deliberately. Pressures were recorded with the Hamilton manometer.[11] All gas analyses were performed with the manometric method of Van Slyke and Neill.[12]

Results. Table I demonstrates that the oxygen contents of coronary venous blood were significantly below those of both right auricular and ventricular blood. The difference between the oxygen contents of peripheral arterial blood, (and consequently of coronary arterial blood), and of coronary venous blood varied from 11.3 to 18.7 vol %, while the total systemic arteriovenous oxygen difference varied from 2.8-7.3 vol %, (Table I). In three cases in which pressures were recorded, the average blood pressure in the coronary sinus was 12 mm Hg.

Summary. The coronary sinus and the middle cardiac vein of man has been successfully catheterized. Work is in progress to utilize this technique in the measurement of coronary blood flow in man.

9 Best and Taylor, 4th Edition, p. 278.
10 Dexter, L., and Sosman, M. C., *Radiology*, 1947, **48**, 441.
11 Hamilton, W. F., Broener, G., and Brotman, I., *Am. J. Physiol.*, 1934, **107**, 427.
12 Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, 1924, **61**, 523.

16050

Effects of Dibenamine on Cardiovascular Actions of Epinephrine, Acetylcholine, Pitressin and Angiotonin in Unanesthetized Dogs.*

W. B. YOUMANS AND V. M. RANKIN.

From the Department of Physiology, University of Oregon Medical School, Portland, Oregon.

Nickerson and Goodman[1] have described adrenolytic actions of N, N-dibenzyl beta-chloroethyl amine (Dibenamine). They found that the compound, when injected slowly intravenously, produced minimal effects upon arterial blood pressure and, within 30 minutes after the injection, the pressor action of epinephrine was blocked in some species and reversed in others. The effects of stimulating various excitatory adrenergic nerves and the excitatory actions of epinephrine upon various effectors were prevented by Dibenamine. On the other hand, the inhibitory actions of epinephrine upon the non-pregnant cat uterus *in situ* and on the isolated small intestine of the rabbit and rat were not prevented by Dibenamine. They also observed that larger doses were required to block the effects of stimulating excitatory adrenergic nerves than were required to block the excitatory effects of injected epinephrine. Cardio-accelerator actions of epinephrine were not blocked by Dibenamine.

The present study is concerned principally with the cardiovascular actions of Dibenamine and with its effects upon the cardiovascular responses to epinephrine, acetylcholine, pitressin, and angiotonin in unanesthetized dogs.

Methods. Unanesthetized animals were trained to lie quietly on the table while a needle was kept in place in the radial vein. Heart rate was determined by counting the apex beat and from continuous electrocardiographic records taken during injection of the 4 test compounds. In one animal the spinal cord had been transected 24 hours previously and blood pressure was recorded from the femoral artery by the use of a mercury manometer.

N, N-dibenzyl beta-chloroethyl-amine hydro-chloride was prepared for injection by dissolving 200 mg in approximately 15 cc of propylene glycol, and this was diluted with an equal amount of water. Injection of the solution was started within 2 to 5 minutes after preparation, and a period of 5 to 20 minutes was required to give the animal the total dose of 200 mg.

Seven dogs weighing between 9 and 13 kg were used. Six of these were intact, and one had the spinal cord transected. The effects of test doses of epinephrine, acetylcholine, pitressin, and angiotonin upon heart rate were recorded before and at stated intervals after administration of Dibenamine. During each series of injections a needle was placed in the radial vein and kept open by injecting small amounts of isotonic saline. At the desired time a syringe containing the exact amount of the compound to be administered was substituted for the syringe containing saline and the compound was injected rapidly. Ample time was allowed for return of the heart rate to the resting level between injections.

Results. A. Effects of Dibenamine on heart rate. Dibenamine alone caused an increase in heart rate in each of the 6 intact animals. The maximal increase above the basal rate ranged from 46% to 95%, and it occurred in 10 to 25 minutes from the beginning of the injection. Usually the rates returned to the resting level within one to 3 hours.

B. Effects of epinephrine on heart rate before and after Dibenamine. Continuous electrocardiographic records were obtained during the test dose of epinephrine (2 cc of a 1-100,-000 dilution) in 4 dogs before and after administration of Dibenamine. The averages of the rates for the 4 animals are graphed in Fig. 1.

The test dose of epinephrine produced a

1 Nickerson, M., and Goodman, L., J. Pharm. and Exp. Therap., 1947, 89, 167.

FIG. 1.

direct accelerator influence of epinephrine and the reflex accelerator influence of the fall in blood pressure. This interpretation is supported by the observation that in an unanesthetized animal, with spinal cord transected at T_{12} so that blood pressure could be recorded from the femoral artery, injection of the test dose of epinephrine, after the animal had been given Dibenamine, caused an immediate fall of 50 mm of mercury in the mean arterial blood pressure. Animals under the influence of Dibenamine also characteristically showed much greater respiratory stimulation after the epinephrine injection than was seen in the unmedicated animals. Presumably, this respiratory stimulation is elicited reflexly from the fall in blood pressure.

The striking cardiac acceleration in response to epinephrine after Dibenamine would seem to indicate that the cardiac effects of epinephrine or of cardio-accelerator nerves are not blocked. However, a large part of this acceleration could be on the basis of decreased tonus of cholinergic cardio-inhibitory nerves.

C. *Effects of Dibenamine on the cardio-accelerator response to acetylcholine.* The brief hypotension produced by intravenous injection of acetylcholine elicits a typical cardio-accelerator response. The acceleration is due largely to reflex activation of adrenergic nerves and to liberation of epinephrine
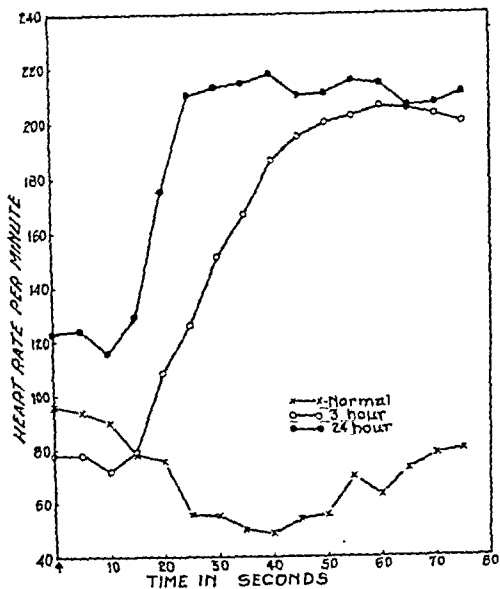
maximal decrease in rate of 45% to 57% in the 4 intact animals before Dibenamine. Since the direct influence of epinephrine upon the sino-auricular node is excitatory, this bradycardia is best explained on the basis of reflex effects elicited by a rise in blood pressure.

Three hours after Dibenamine had been administered the average heart rate was 19 beats per minute slower than the average of the control rates. At this time the test dose of epinephrine produced an acceleration of 97% to 208% above the rate preceding the injection. Acceleration was clearly evident within 20 seconds after the injection of epinephrine and progressed smoothly to a plateau at about 50 seconds. It was still maintained at 75 seconds. The effects of epinephrine on the heart rate 24 hours after Dibenamine were similar to the effects seen 3 hours after Dibenamine.

The reversal of the effect of the test dose of epinephrine on the heart rate of unanesthetized animals by Dibenamine may be readily explained if it is considered that epinephrine causes a fall in blood pressure in the unanesthetized animals after they have received Dibenamine. If such is the case, a smooth cardiac acceleration would be expected because of the combination of the
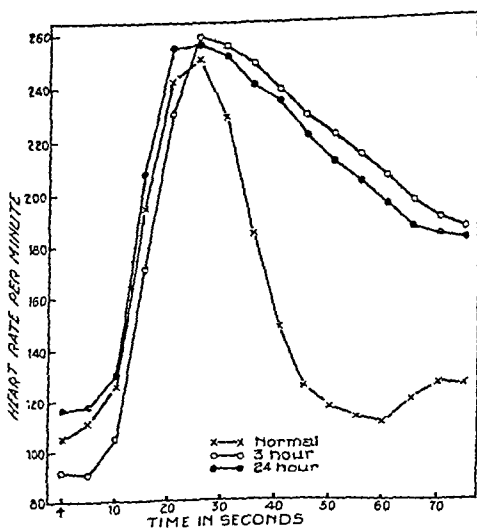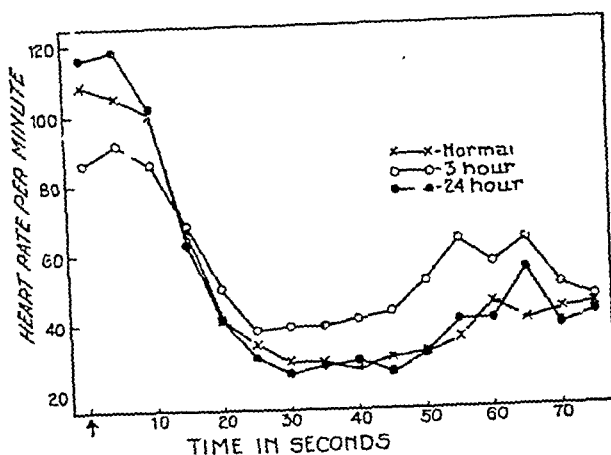

FIG. 2.

FIG. 3.

from the adrenal medulla.[2] Five animals were given a test dose of 1 mg of acetylcholine at 3 hours and again at 24 hours after the administration of Dibenamine. In Fig. 2 a curve is shown which illustrates the average of the cardio-accelerator responses to the test dose of acetylcholine in 23 animals. The other 2 curves in Fig. 2 illustrate the averages of the cardio-accelerator response to acetylcholine in 4 animals at 3 hours and at 24 hours after Dibenamine. The degree of acceleration was at least as great after Dibenamine as before. In both the normal animals and in those under the influence of Dibenamine, the maximum increase in heart rate in response to acetylcholine occurred at 25 seconds. In the normal animals the rate fell off rapidly, to reach a resting level within 50 to 60 seconds, but in the animals under Dibenamine the return to the resting level was quite delayed. The persistence of the fast rate would indicate that Dibenamine interferes with the restoration of the blood pressure to the normal level after a test dose of acetylcholine. This result is compatible with the interpretation that vasoconstrictor mechanisms are impaired by Dibenamine while cardio-accelerator mechanisms are relatively intact.

D. *Effects of Dibenamine upon the cardio-inhibitory response to pitressin and angiotonin.* The effects of Dibenamine on the pressor actions of pitressin and angiotonin were tested in 3 dogs which had received sodium pentobarbital. At the time when the pressor action of epinephrine was reversed, there was no interference with the pressor actions of either pitressin or angiotonin. In one animal under sodium pentobarbital, neurogenic hypertension was produced by sino-aortic denervation. When Dibenamine was injected in this animal, it produced a profound fall in blood pressure.

In unanesthetized dogs pitressin and angiotonin produce a pronounced reflex bradycardia during the rise in blood pressure resulting from their vasoconstrictor action.[3] If the vasoconstrictor action of these compounds is not blocked by Dibenamine, they would be expected to produce the typical cardio-inhibitory response. The effects of a test dose of 1½ pressor units of pitressin before and after Dibenamine were studied in 4 dogs, and the effects of a test dose of 20 pressor units of angiotonin were studied in 3 dogs. The averages of the cardio-inhibitory responses to pitressin in 4 dogs at 3 hours and at 24 hours after Dibenamine are shown in Fig. 3, and these are compared with a curve showing the averages of 14 normals. The results of a typical experiment with angiotonin are graphed in Fig. 4.

From these results it is evident that Dibena-

[2] Youmans, W. B., Aumann, K., Haney, H., and Wynia, F., *Am. J. Physiol.*, 1940, **128**, 467.

[3] Haney, H. F., Lindgren, A. J., Karstens, A. I., and Youmans, W. B., *Am. J. Physiol.*, 1943, **139**, 675.
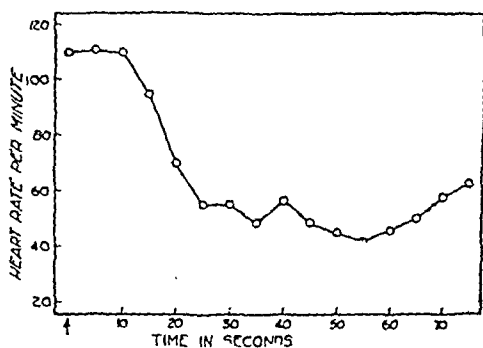
FIG. 4.

mine does not alter the cardio-inhibitory responses produced by the injection of pitressin and angiotonin. This would indicate that, as in the anesthetized animals, Dibenamine does not impair the vasoconstrictor actions of these compounds.

Summary and Conclusions. The actions of Dibenamine on the cardiovascular adjustments caused by epinephrine, acetylcholine, pitressin, and angiotonin have been studied in unanesthetized dogs. Dibenamine produced an increase in heart rate which persisted for one to 3 hours.

A test dose of epinephrine which regularly caused reflex cardiac slowing in normal unanesthetized dogs produced severe cardiac acceleration after administration of Dibenamine. This acceleration is attributable to the direct stimulatory action of epinephrine on the sino-auricular node and to reflex acceleration from a fall in blood pressure.

The compensatory cardiac acceleration produced by injection of a test dose of acetylcholine was undiminished and prolonged in unanesthetized animals under the influence of Dibenamine. This result is compatible with the interpretation that the vasoconstrictor mechanisms are impaired while the cardio-accelerator mechanisms are relatively intact.

The severe cardiac inhibition produced by pitressin and angiotonin in unanesthetized dogs, which is attributable to reflexes elicited by the rise in arterial blood pressure subsequent to vasoconstriction, was not prevented by Dibenamine.

The results of these experiments on unanesthetized dogs are in accord with the interpretations of Nickerson and Goodman[1] concerning the sites of action of Dibenamine.

16051

Diabetogenic Effect of Two Synthetic Estrogens in Force-Fed, Alloxan-Diabetic Rats.

DWIGHT J. INGLE AND JOHN A. HOGG.

From the Research Laboratories, The Upjohn Company, Kalamazoo, Mich.

Ingle[1] has reported on the diabetogenic effect of diethylstilbestrol, dihydrostilbestrol, estradiol and equilin in the force-fed, partially depancreatized rat. More recently it was shown by Ingle, Nezamis and Prestrud[2] that diethylstilbestrol will intensify the glycosuria of rats having alloxan diabetes when the food intake is kept constant by forced feeding. The present study was a partial test of the hypothesis that compounds which are estro-

genic are also diabetogenic in the rat. Substance I (2,4-di[p-hydroxyphenyl]-3-ethyl hexane) was described by Blanchard, Stuart and Tallman[3] and Substance II (1,2-dimethyl-2-carboxy-7-methoxy - 1,2,3,4,9,10-hexahydrophenathrene) was described by Hogg.[4]

Each of these two compounds belongs to a chemically different series of synthetic estrogens than any of the substances which have previously been examined for diabetogenic

1 Ingle, D. J., *Endocrinology*, 1941, **29**, 838.
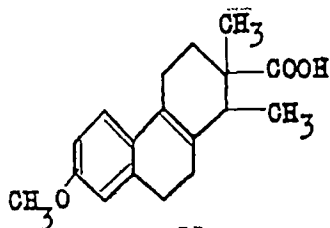
2 Ingle, D. J., Nezamis, J. E., and Prestrud, M. C., *Endocrinology*, 1947, **41**, 207.

3 Blanchard, E. W., Stuart. A. H., and Tallman, R. C., *Endocrinology*, 1943, **32**, 307.

4 Hogg, J. A., *J. Am. Chem. Soc.*, in press.
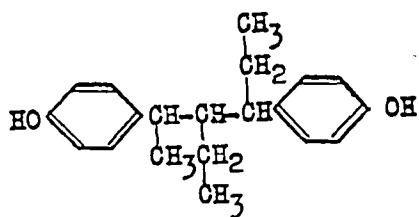
I

II

activity. Each compound was found to intensify the glycosuria of the alloxan-diabetic rat.

Methods. Male rats of the Sprague-Dawley strain were maintained on Purina Dog Chow until they reached a weight of 310 g. They were then fed a medium carbohydrate diet made according to Table I. During the administration of alloxan all of the rats ate the diet *ad libitum.* Alloxan was injected subcutaneously in doses of 25 mg every other day until glycosuria appeared. After a diabetic state was established the animals were placed in metabolism cages and were force-fed by stomach tube each morning (8:30 to 9:15 a. m.) and afternoon (4:15 to 5:00 p. m.). The techniques and the diet were modifications of those described by Reinecke, Ball and Samuels.[5] During the period of adaptation to
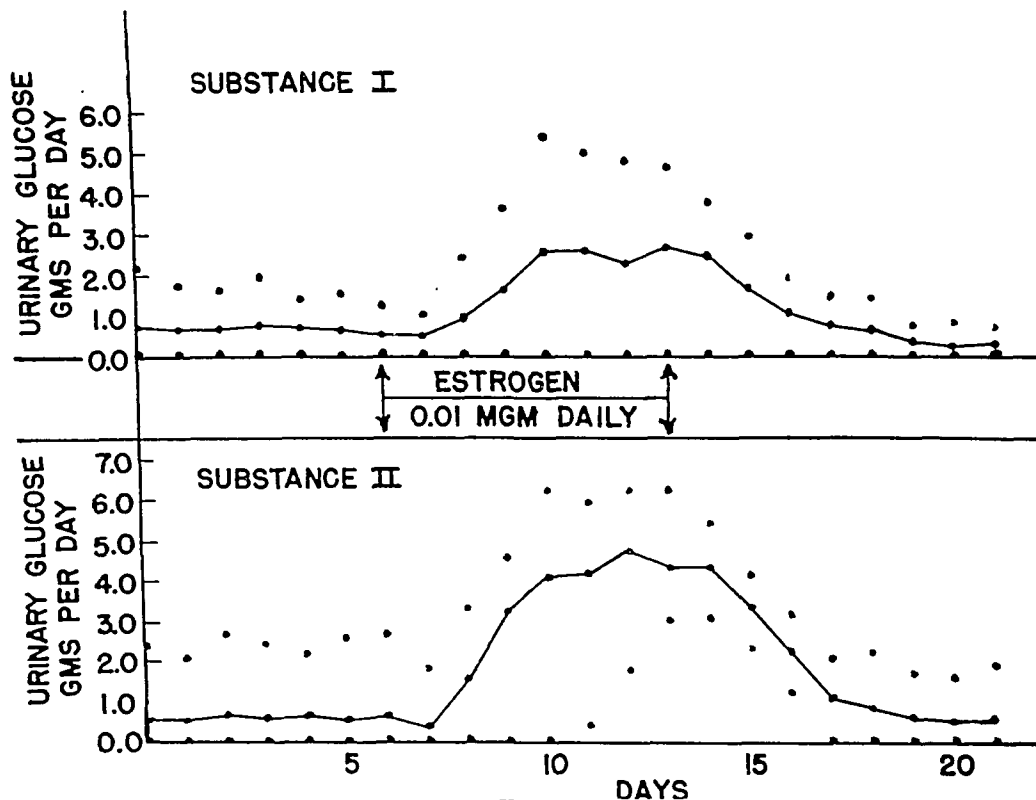

FIG. 1.
The effect of two synthetic estrogens upon the level of urinary glucose in alloxan-diabetic rats. Averages (solid line) and range of individual values. Six rats were tested in each of the two experiments.

TABLE I.
Composition of Fluid Diet.

Constituent		
Cellu flour (Chicago Dietetic Supply)	120	g
Osborne and Mendel salt mixture	40	
Dried yeast (Pabst)	100	
Wheat germ oil	10	
Cod liver oil	10	
Mazola oil	200	
Vit. K (2-methyl-1-4-naphthaquinone)	100	mg
Casein (Labco)	160	
Starch	200	
Dextrin	190	
Sucrose	200	
Egg albumin (Merck)		
Water to make total volume of	2000	cc

forced feeding, the amount of diet was increased gradually to prevent the development of "food-shock." The animals were brought to a full feeding of 26 cc of diet per rat per day on the fifth day.

The animals were maintained in an air-conditioned room in which the temperature was maintained at 74-78°F and the humidity at 30-35% of saturation. Twenty-four-hour samples of urine were collected at the same hour (8:00 to 8:30 a. m.) and were preserved with thymol. Urine glucose was determined by the method of Benedict.[6]

The estrogens were made up in vegetable oil solution and were each given by subcutaneous injection once daily in a volume of 0.1 cc. Substance I (Schieffelin) was purchased on the market. Substance II was prepared by one of us (JAH) by laboratory synthesis.

Experiments and Results. Twelve rats having mild alloxan diabetes were used in these experiments. The animals were observed for a period of 3 to 4 weeks before the tests of the estrogens were started. In experiment 1, 6 rats were injected with 0.01 mg of Substance I per day for 7 days. Two of the rats were free from glycosuria during the control period. Five of the 6 rats responded to the injections of Substance I by an increase in the level of urinary glucose. When the injections were stopped the level of glycosuria

decreased. The sixth rat did not excrete glucose during any phase of the experiment.

In Experiment 2, 6 rats were injected with 0.01 mg of Substance II per day for 7 days. Three of the rats were free from glycosuria during the control period, but all of the animals showed a significant increase in urinary glucose during the injection period. When the injections were stopped the glycosuria decreased in all of the animals and disappeared entirely in 4 of the 6 animals.

The data on urinary glucose are summarized in Fig. 1.

Discussion. The results of these experiments support our tentative hypothesis that compounds which are estrogenic also have the property of intensifying the glycosuria of mildly diabetic, force-fed rats. Other hormonal substances, especially those of the adrenal cortex and the anterior pituitary, are diabetogenic in the rat although they lack estrogenic activity. Of the polycyclic compounds which have been tested, only those having estrogenic activity or those having adrenal cortical activity have been found to be diabetogenic. The mechanism of diabetogenic activity in these compounds and its relationship to the role of the hormones in body economy are unknown.

These data are relative to the conclusion of Janes and Dawson[7] that estrogens are not diabetogenic in the rat and support the findings of Ingle, Nezamis and Prestrud[2] who found that diethylstilbestrol does intensify the glycosuria of the alloxan-diabetic rat just as in the partially depancreatized rat (Ingle[1]). There are two essential conditions for the exacerbation of diabetes by estrogens in the rat. First, the test animal should be mildly diabetic rather than severely diabetic. Second, the food intake must be sustained by forced feeding, otherwise the diabetogenic effect of the estrogen may be partially or completely *masked by the concomitant decrease in voluntary food intake.* These essential conditions were not observed in the study by Janes and Dawson.[7]

Summary. Male rats having mild alloxan

5 Reinecke, R. M., Ball, H. A., and Samuels, L. T., PROC. SOC. EXP. BIOL. AND MED., 1939, 41, 44.

6 Benedict, S. R., J. A. M. A., 1911, 57, 1193.

7 Janes, R. G., and Dawson, H., *Endocrinology*, 1946, 38, 10.

diabetes were used as test animals in this study. They were force-fed a medium carbohydrate diet. Two synthetic estrogens were tested, each of which represents a chemically different series of compounds than have previously been tested for diabetogenicity. Substance I was 2,4-di(p-hydroxyphenyl)-3-ethyl hexane, and Substance II was 1,2-dimethyl-2-carboxy-7-methoxy - 1,2,3,4,9,10-hexahydro-

phenathrene. Substance I caused exacerbation of the diabetes in 5 out of 6 test animals. The sixth rat did not have a spontaneous glycosuria and failed to respond to the estrogen. Substance II intensified the glycosuria in all 6 test animals and appeared to be the more potent of the two compounds. When the injections were stopped the glycosuria decreased to its pre-injection level or below.

16052

BAL Inhibition of Mercurial Diuresis in Congestive Heart Failure.[*]

RALPH M. SUSSMAN AND JEROME A. SCHACK.   (Introduced by E. H. Fishberg.)

From the Medical Service of Beth Israel Hospital, New York City.

This study was undertaken to evaluate the effect of 2,3 dimercaptopropanol[1] (dithiopropanol),—commonly known as British Anti-lewisite or BAL—upon the diuresis induced by the organic mercurials with the view of gaining further information regarding the mechanism of mercurial diuresis, as well as controlling some of the untoward sensitivity reactions[2,3] encountered when these substances are employed. 1 - (methoxy - oxymercuri - propyl) - 3 - succinylurea—generally known as Mercuhydrin—which contains 39 mg of mercury in organic combination with 48 mg of theophyllin per cc of aqueous solution, was the sole mercurial studied. The preparation known as BAL in oil (10% 2,3 dimercaptopropanol in benzyl benzoate and peanut oil) was used.

The subjects selected were cardiac patients in chronic congestive failure who were known to respond to organic mercurial injection with

diuresis and weight loss. The experience with 7 patients comprises this report. Five patients had hypertensive arteriosclerotic heart disease; one was a rheumatic cardiac and the other suffered from heart failure of undetermined etiology. Regular sinus rhythm was present in 6 of 7 of these subjects at the time of the study.

So far as feasible, the patients were maintained on the usual therapeutic cardiac regimen consisting of incomplete bed rest, salt-poor diet, containing less than 2 g of NaCl in 24 hours, no fluid restriction, and in most instances the administration of some digitalis preparation.

When it appeared that they had attained a relatively constant weight under such management, BAL in oil was introduced for a period of 24 to 36 hours. It was administered intramuscularly every 6 hours in doses of 2.5 mg per kilo of body weight. After receiving BAL in oil for 24 hours, Mercuhydrin was injected intramuscularly in 2 cc doses. The BAL in oil was then continued for a period of 12 hours.

Table I contrasts the weight changes occurring in patients receiving BAL in oil for about 24 hours, prior to the administration of Mercuhydrin with those who received the mercurial only.

The individaul weight loss 48 hours after

1 Peters, R. A., Stocken, L. A., and Thompson, R. H. S., *Nature*, 1945, 156, 616.

2 Brown, G., Friedfeld, L., Kissin, M., Modell, W., and Sussman, Ralph M., *J. Am. Med. Assn.*, 1942, 119, 1004.

3 Long, W. K., and Faran, A., *Science*, 1946, 104, 220.

TABLE I.
Composition of Fluid Diet.

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forced feeding, the amount of diet was increased gradually to prevent the development of "food-shock." The animals were brought to a full feeding of 26 cc of diet per rat per day on the fifth day.

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decreased. The sixth rat did not excrete glucose during any phase of the experiment.

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5 Reinecke, R. M., Ball, H. A., and Samuels, L. T., PROC. SOC. EXP. BIOL. AND MED., 1939, 41, 44.

6 Benedict, S. R., J. A. M. A., 1911, 57, 1193.

7 Janes, R. G., and Dawson, H., Endocrinology, 1946, 38, 10.

16053 P

Isolation of Western Equine Encephalomyelitis Virus from Tropical Fowl Mites, *Liponyssus bursa* (Berlese).*

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At the present time the exact mode of transmission of western equine encephalomyelitis is far from established, and the mechanism by which the causative agent persists from year to year in a given area has not been fully explained. Numerous attempts have been made to determine reservoir hosts and vectors of the disease. Hammon and his associates[1] demonstrated that a large per cent of both domestic and wild birds possess neutralizing antibodies for this virus suggesting that the reservoir host might be one closely associated in some manner with domestic fowl. It seemed likely, as pointed out by Smith and her associates,[2] that some blood-sucking vector which does not necessarily bite man was transmitting the disease to fowl. These investigators recently reported the isolation of the St. Louis encephalitis virus from naturally infected chicken mites, *Dermanyssus gallinae* (DeGeer), in the St. Louis area where the disease is endemic. Later, they reported several additional isolations from chicken mites, and demonstrated transovarian transmission of the virus from naturally infected female mites.[3] Shortly thereafter, Sulkin[4] reported the isolation of the Western equine virus from naturally infected chicken mites in the Dallas

area during the epidemic year of 1944. During the following non-epidemic year additional attempts to demonstrate this virus in chicken mites and fowl ticks collected in this identical locality and other areas in Texas, were unsuccessful. Numerous attempts by Hammon and his associates[5] to demonstrate virus in this ectoparasite have been negative. Since the possible role of the chicken mite in the transmission and effective perpetuation of the virus encephalitides is not yet clarified, it was considered desirable to continue the search for a reservoir among other avian blood-sucking ectoparasites.

Recently Reeves and his collaborators[6] reported recovery of the Western equine virus from wild bird mites, *Liponyssus sylviarum* (Canestrini and Fanzago), in Kern County, California. The present report concerns an additional isolation of the Western equine virus from similar mites collected in Dallas County, Texas from a nest containing two live nestling English sparrows, *Passer domesticus* (Linn.).† The nest was brought into the laboratory on July 9, 1947. A layer of white absorbent cotton was placed over the top, and the entire nest was then placed in a covered glass container. On the following day, mites identified as *Liponyssus bursa* (Berlese),† were collected from the cotton and·the underside of the lid, and were tested

1 Hammon, W. McD., Lundy, H. W., Gray, J. A., Evans, F. C., Bang, F., and Izumi, E. M., *J. Immunol.*, 1942, **44**, 75; Hammon, W. McD., Reeves, W. C., and Irons, J. V., *Texas Rep. Biol. and Med.*, 1944, **2**, 366.

2 Smith, M. G., Blattner, R. J., and Heys, F. M., *Science*, 1944, **100**, 362.

3 Smith, M. G., Blattner, R. J., and Heys, F. M., Proc. Soc. Exp. Biol. and Med., 1945 **59**, 136; *J. Exp. Med.*, 1946, **84**, 1.

4 Sulkin, S. E., *Science*, 1945, **101** 381.

5 Hammon, W. McD., and Reeves, W. C., *Am. J. Pub. Health*, 1945, **35**, 994.

6 Reeves, W. C., Hammon, W. McD., Furman, D. P., McClure, H. E., and Brookman, B., *Science*, 1947, **105**, 411.

† The authors are indebted to Dr. E. P. Cheatum, Department of Biology, Southern Methodist University, for confirming the identifications, and to George L. Carpenter, Typhus Control Supervisor, United States Public Health Service, for collecting specimens for study.

TABLE I.
Weight Change.

Subject	BAL with Mercurial		Mercurial	
	24 hr	48 hr	24 hr	48 hr
J.D.	— ¾	+ ¾	—3¼	—5¼
S.S.	+ ½	+1½	—4¼	—3¾
H.G.	0	+1	—4	—3½
E.G.	— ½	— ¼	—3½	
F.S.	— ¾	— ½	—2¾	—3¾
G.N.	0	+1	—3¾	—2¾
N.R.	— ¼	+1	—1¾	—3¼
Avg	— ¼	+ ⅗	—3⅓	—3⅔

the administration of the mercurial, alone, was somewhere between 2¾ and 5¼ lb, whereas, patients receiving BAL in oil with the mercurial, in most instances gained weight, only 2 having lost ½ lb or less.

Some observations made by varying the period of BAL administration preliminary to mercurial injection indicate that at least 4 doses of 2.5 mg per kilo body weight injected at intervals of 6 hours must be given prior to the introduction of the 2 cc of the mercurial in order to inhibit diuresis completely. It is also evident from this investigation that within 48 hours after the last injection of BAL in oil, diuresis can again be anticipated from another adequate injection of mercurial. Patients who were observed for several days following the cessation of BAL in oil injections displayed no evidence of diuresis from the mercurial administered simultaneously with the dithiopropanol. It may, therefore, be concluded that BAL in oil completely annuls the diuretic action of the mercury and does not merely delay it.

Notes made on the side-reactions experienced with BAL in oil coincide with earlier pharmacologic observations. Thus, transient rise in blood pressure, local reaction, nausea, vomiting, warmth, paraesthesias and chill were all observed.[4]

In 2 hypertensive patients, alarming elevation of systolic and diastolic blood pressures coincided with the administration of BAL in oil and persisted for at least 4 hours. In one patient the symptoms were interpreted as

being due to "hypertensive encephalopathy".[5]

Considerable local pain was experienced at the site of injection and the administration of 0.5 cc of 1% procaine in the syringe with each injection relieved one patient considerably without in any way diminishing the antidiuretic effect of the dithiopropanol.

No cases of organic mercurial sensitivity were available at the time of study, so that no remarks regarding the response of such patients to BAL can be made.

Unless differences in species reaction occur, it is thought that the mechanism of BAL inhibition of mercury diuresis results from the formation of a stable mercury-thiol complex,[6,7] binding free mercury, and that BAL is not, itself, an anti-diuretic substance.

These findings further substantiate the notion that the diuresis from organic mercurials is due to the systemic effect of free mercury.

Since this study was completed, an important communication from Handley and La Forge has appeared and has established that BAL injected intravenously into dogs inhibits mercurial diuresis in that animal.[8] Their findings in the normal dog are in complete accord with our observations in humans suffering from heart failure.

Conclusions. 1. BAL in oil completely annuls the diuretic effect of the organic mercurial known as Mercuhydrin.

2. A period of priming with BAL in oil was necessary to inhibit the diuresis completely.

3. Procaine may be added to BAL in oil without in any way affecting the anti-diuretic effect.

4. BAL in oil may intensify pre-existing hypertension in the presence of heart failure.

4 Modell, Walter, Gold, Harry, and Cattell, McKeen, *J. Clin. Invest.*, 1946, **25**, 480.

5 Fishberg, A. M., *Hypertension and Nephritis*, Chapter X, Lea & Febiger, 4th edition, 282.

6 Barron, E. S. G., and Kalnitsky, G. (quoted by Longcope, W. T., and Luetscher, J. A.), *J. Clin. Invest.*, 1946, **25**, 557.

7 Gilman, Alfred, Allen, Roberta P., Philips, Frederick S., and St. John, Ellen, *J. Clin. Invest.*, 1946, **25**, 549.

8 Handley, Carroll A., and La Forge, Marguerite, PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 74.

16054

Indoleacetic Acid and Growth of Bacteria with Varying Requirements for Nicotinic Acid and Tryptophane.

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An antagonism between indole-3-acetic acid, and nicotinic acid and/or tryptophane, was suggested by the data of Kodicek, Carpenter, and Harris[1] as a possible explanation for the role of corn in the etiology of pellagra. It was thought of interest to study the relationship between these compounds in certain bacteria whose nutritional requirements are relatively well known, and which can be grown in chemically defined media.

The following organisms were selected (numbers in parentheses refer to American Type Culture Collection listings): Requiring both nicotinic acid and tryptophane: *Lactobacillus casei* (7469), *Streptocococcus faecalis* R (8043), and *Lactobacillus arabinosus* 17-5 (8014). Shankman *et al.*, state[2] that the nicotinic acid requirement of the latter organism is abolished upon long incubation. Requiring nicotinic acid alone: *Staphylococcus aureus*; requiring neither: *Escherichia coli* and *Aerobacter aerogenes*.

After this work was initiated, Dubos[3] reported that indoleacetic acid in concentrations of 0.01 to 0.10 mg/ml inhibited *Mycobacterium tuberculosis* (Human strain), certain streptococci, and *Shigella paradysenteria* (Sonne) grown in enzymatic casein hydrolyzate-yeast extract medium at pH 6.0. This inhibition was reported to be relieved by concentrations of added tryptophane ten times that of the inhibitor.

Experimental. The lactic acid bacteria and *S. aureus* were grown on either the amino acid medium of Stokes *et al.*,[4] with sodium citrate substituted for sodium acetate, or, in most cases, on the same medium with acid-hydrolyzed casein substituted for the amino acid medium. These variations produced no significant alteration in results. *E. coli* and *Aerobacter aerogenes* were grown on MacLeod's[5] medium of glucose, ammonium sulfate, asparagine, and salts. Following the appearance of Dubos' report, a non-pathogenic strain of Mycobacterium tuberculosis (American Type Culture Collection No. 607) was tested, using Long's synthetic medium.

In all cases, indoleacetic acid, nicotinic acid, and tryptophane were adjusted to the pH of the respective media, and added as required. The medium was autoclaved following these additions. Except in the case of *M. tuberculosis*, where a small fragment of mycelium was employed for inoculation, the inoculum consisted of one drop of a saline suspension of organisms which had been washed with saline and then suspended in 2.5 times the original culture volume.

For lactic acid bacteria, growth was measured after 48 to 72 hours incubation at 37°C by titration with 0.1 N NaOH to pH 6.8 on a Berkman pH meter. Growth of *M. tuberculosis* was measured, after either 3 or 6 days incubation, by filtering, washing, drying, and weighing the mycelium. Growth of the other organisms was measured as turbidity (expressed as optical density) in the Evelyn Photoelectric Colorimeter after 24 to 48 hours incubation. In none of these cases did variations in incubation time significantly alter results.

Results. The results of these experiments are indicated in Table I. Each indicated value

[1] Kodicek, E., Carpenter, K. J., and Harris, L. J., *The Lancet*, 1946, **251**, 491.

[2] Shankman, S., Camien, M. N., Block, H., Merrifield, R. B., and Dunn, M. S., *J. Biol. Chem.*, 1947, **168**, 23.

[3] Dubos, R. J., Proc. Soc. Exp. Biol. and Med., 1946, **63**, 317.

[4] Stokes, J. L., Gunness, M., Dwyer, I. M., and Caswell, M. C., *J. Biol. Chem.*, 1945, **160**, 35.

[5] MacLeod, C. M., *J. Exp. Med.*, 1940, **72**, 217.

in 3 pools for the presence of a neurotropic virus. The three pools, each containing approximately 300 mites (adults and nymphs), were aspirated into small vials as described elsewhere[7] and allowed to remain overnight. Each pool was ground in a mortar with 2.0 cc of nutrient broth, and then centrifuged at 5000 r.p.m. for 15 minutes in an angle centrifuge. Approximately 0.3 cc of the supernatant fluid, which was cultured aerobically and anaerobically, was injected intraperitoneally into each of 5 Swiss mice. Pool No. 1 was inoculated into 8-day-old mice and pools No. 2 and No. 3 into 12-day-old mice. The supernatant fluids did not contain enough bacteria to affect the animals. An agent later identified as western equine encephalomyelitis virus was recovered from pool No. 1 and after 4 serial passages in Swiss mice, filtration experiments (Berkefeld N filter) were conducted with the pooled brains of mice showing neurological symptoms. Identification of a filterable agent of the sixth passage as the Western type of equine encephalomyelitis virus was made by neutralization tests with serum of rabbits immunized to known strains of Western and Eastern equine virus and St. Louis encephalitis virus. The virus recovered from the sparrow mites was neutralized by the Western equine immune serum but was not affected by the serum of rabbits immunized to the Eastern strain of equine virus or the virus of St. Louis encephalitis. With each passage, before and after the filtration experiment, aerobic and anaerobic cultures were made and brain tissue from animals showing central nervous system symptoms were examined for histopathological evidence of encephalitis. Microscopic sections of a mouse and a guinea pig brain from the second intracerebral passage showed histopathological evidence characteristic of encephalitis. Pools No. 2 and No. 3 of mites

collected from the same source but injected into older mice yielded negative results. All attempts to isolate a filterable virus from 11 additional pools of specimens of wild bird mites yielded negative results. Additional attempts to demonstrate virus in chicken mites were likewise negative. Studies are still in progress to determine whether or not hereditary transmission of the Western equine virus in the chicken mite can be effected under experimental conditions. Similar studies will also be made with the wild bird mite.

Thus, in addition to the well documented evidence incriminating the mosquito in the transmission of western equine encephalomyelitis,[5,8] it is becoming increasingly apparent that other blood-sucking arthropods may be concerned in the transmission of the virus of equine encephalomyelitis.

Summary. The Western type of equine encephalomyelitis virus has been isolated from wild bird mites, *Liponyssus bursa* (Berlese), collected in the Dallas County, Texas area. At the present time the epidemiological role played by the wild bird mite and the chicken mite, *Dermanyssus gallinae* (DeGeer), from which the Western equine virus had been previously isolated, is unknown. The purpose of this report is to emphasize the importance of virus isolations from similar bird mites in two widely separated areas.

ADDENDUM: Two months after the Western equine virus was isolated from the tropical bird mites collected in Dallas County, the Eastern equine virus was recovered from a horse brain submitted to us from Jefferson County, Texas, where a widespread epizootic was occurring. This would indicate that both the Western and Eastern equine types of encephalomyelitis were active in Texas at about the same time.

7 Wisseman, C. L., Jr., and Sulkin, S. E., *Am. J. Trop. Med.*, 1947, **27**, 463.

8 Hammon, W. McD., Reeves, W. C., Brookman, B., and Izumi, E. M., *J. Inf. Dis.*, 1942, **70**, 263.

TABLE II.
Effect of Indoleacetic Acid on Growth of *M. tuberculosis*.

IAc mg/ml	T mg/ml	NA mg/ml	Growth mg dried mycelium
0	0	0	.167
0	0	2	.144
0	2	0	.157
.2	0	0	.151
.2	0	2	.167
.2	2	0	.137

no relationship to the nicotinic acid or tryptophane requirements of the organism, and is not relieved, in the organisms studied, by either of the latter two substances. In fact, the organism most strongly inhibited by indoleacetic acid, *Aerobacter aerogenes*, requires neither tryptophane nor nicotinic acid for growth. It is interesting to note that *E. coli*, for which stimulation by indoleacetic acid at levels up to one γ/ml in a medium containing 0.1 mg/ml of tryptophane has been reported,[6] was unaffected by the lower concentration of indoleacetic acid used in this study, and was inhibited by the higher concentration. *S. aureus* showed no inhibition at the highest concentration of indoleacetic acid used.

In certain cases, particularly *L. arabinosus* and *L. casei*, a stimulatory effect at 0.1 mg·ml, and sometimes at 1 mg ml, levels of indoleacetic acid was observed when the concentration of tryptophane was limiting: this stimulation disappeared at adequate levels of tryptophane regardless of nicotinic acid content. The latter organisms are not specific in their response to tryptophane,[7,8] since indole and anthranilic acid have been reported to have tryptophane activity. However, indoleacetic acid was stated to be inactive. It should be noted, however, that the latter studies[7,8] measured the response of these organisms to tryptophane derivatives in media completely devoid of tryptophane: it is possible that indoleacetic acid may stimulate only in the presence of small amounts of the amino acid.

It is realized that Dubos obtained relief of inhibition at concentrations of added tryptophane 10 times that of inhibitor, and that this ratio was not used in the present experiments. Further, Dubos used a basal medium already containing tryptophane in the enzymatic casein hydrolyzate and yeast extract. Because of these facts, our results and those of Dubos are not strictly comparable. Since our inhibition was generally obtained with one mg ml of indoleacetic acid, using Dubos' ratio would not only have led to difficulties due to solubility, but would have produced such a high concentration of tryptophane as to make interpretation of results difficult. If a true metabolite-antagonist relationship exists between indoleacetic acid and nicotinic acid-tryptophane, it is a relatively unusual phenomenon that far greater concentrations of metabolite than of the antagonist are required to relieve inhibition. This fact, as well as the lack of relationship between nicotinic acid and tryptophane requirements of organisms and their susceptibility to indoleacetic acid inhibition, tend to detract from the probability of a true metabolite-antagonist relationship. A recent report by Krehl, Carvalho, and Cowgill,[9] failing to confirm the results of Kodicek *et al.*,[1] should be noted. It would appear that a metabolite-antagonist relationship between indoleacetic acid and nicotinic acid or tryptophane is not an invariably occurring biological phenomenon, nor is there any evidence indicating the mechanism of this reaction where it does occur.

Summary and Conclusions. 1. A number of organisms whose tryptophane and nicotinic acid requirements are known were tested for possible inhibition by indoleacetic acid and reversal by nicotinic acid and tryptophane in chemically defined media.

2. *Escherichia coli, Lactobacillus arabinosus 17-5, L. casei, Streptococcus faecalis R,* and *Aerobacter aerogenes* were inhibited by concentrations of indoleacetic acid of one mg/ml Occasionally slight inhibitions were noted at 0.1 mg/ml levels.

6 Ball, E., *J. Bact.*, 1938, 36, 559.

7 Snell, E. E., *Arch. Biochem.*, 1943, 2, 389.

8 Greene, R. D., and Black, A., *J. Biol. Chem.*, 1944, 155, 1.

9 Krehl, W. A., Carvalho, A., and Cowgill, G. R., *Fed. Proc.*, 1947, 6, 413.

TABLE I.
Effect of Indoleacetic Acid, Nicotinic Acid, and Tryptophane upon Certain Bacteria.

Organism	Exp. No.	IAc (mg/ml)	Growth with indicated concentrations of NA and T (mg/ml)			
			T=0.3 NA=0.01	T=0.3 NA=10	T=30 NA=0.01	T=30 NA=10
Streptococcus fæcalis R	1	0	3.8	5.5	3.8	5.4
Growth measured as ml of		0.1	3.4	5.3	3.4	5.2
0.1 N NaOH		1.0	2.5	—	2.5	3.8
	2	0	4.8	5.2	3.3	5.5
		0.1	4.4	5.0	3.4	4.9
		1.0	3.6	—	—	4.7
	3	0	2.7	3.0	3.5	7.8
		0.1	2.5	2.8	3.3	5.7
		1.0	2.4	3.1	3.1	5.6
	4	0	2.4	2.9	3.5	7.0
		0.1	1.7	2.6	3.4	6.4
		1.0	2.0	2.0	2.7	4.9
Lactobacillus arabinosus 17-5 (8014)	1	0	2.2	2.7	6.0	8.2
Growth measured as ml of		0.1	2.6	3.2	5.8	8.4
0.1 N NaOH		1.0	2.7	5.1	4.2	3.1
	2	0	2.0	2.9	3.2	9.9
		0.1	1.8	3.0	3.2	10.1
		1.0	2.4	2.8	1.4	4.4
Lactobacillus casei (7469)	1	0	2.9	2.9	6.6	—
Growth measured as ml of		0.1	3.1	3.3	6.3	7.8
0.1 N NaOH		1.0	—	4.7	4.0	6.1
	2	0	2.6	3.8	5.1	7.3
		0.1	3.1	3.8	3.0	7.5
		1.0	2.8	—	1.0	3.5
Staphylococcus aureus	1	0	0.31	0.30	—	0.31
Growth measured as optical density		0.1	0.35	0.39	—	0.41
		1.0	0.31	—	—	0.33
	2	0	0.28	0.36	0.33	0.42
		0.1	0.34	0.37	0.32	0.40
		1.0	0.30	0.39	0.32	0.43
Escherichia coli	1	0	0.18	0.17	0.15	0.17
Growth measured as optical density		0.1	0.18	0.17	0.17	0.17
		1.0	0.13	0.10	0.11	0.12
Aerobacter aerogenes	1	0	0.52	0.52	—	0.51
Growth measured as optical density		0.1	0.40	0.53	—	0.50
		1.0	0.13	0.14	—	0.14
	2	0	0.18	0.16	—	0.23
		0.1	0.17	0.14	—	0.17
		1.0	0.10	0.11	—	0.10

IAc = Indole-3-acetic acid.
T = *l*-tryptophane.
NA = Nicotinic acid.

for growth is based upon duplicate measurements.

In addition to the data given in Table I, *L. arabinosus* was grown on the basal medium supplemented with 1.0 mg/ml of nicotinic acid, and 1.0 mg/ml of indoleacetic acid. Varying amounts of tryptophane, from 1 γ to 1 mg/ml, were added, with no relief of inhibition.

In the case of *M. tuberculosis*, the results shown in Table II were obtained after 6 days incubation. Similar results, at lower levels of growth, obtained after 3 days incubation.

Discussion. From the results of these experiments, it can be seen that although indoleacetic acid may act as an inhibitor at relatively high concentrations (1 mg/ml), and, sometimes, to a smaller extent, at concentrations of 0.1 mg/ml, this inhibition appears to bear

TABLE I.

Effect of Hetrazan on Experimental Trichinosis as Determined by the Number of Adult and Larval Trichina Recovered from Rats. Six Rats Used in Each Experiment.

No. of infective larvae fed per rat	Amt. of drug given orally per rat (mg per kilo wt. %, I.D.)	Length of treatment, days	Days after feeding infective larvae when killed					
			5		10		30	
			Avg No. of worms recovered from intestine	Avg No. of larvae recovered from muscle	Avg No. of worms recovered from intestine	Avg No. of larvae recovered from muscle	Avg No. of worms recovered from intestine	Avg No. of larvae recovered from muscle
1,000	300	5	91	0				
" "	0	10	481	0				
" "	300	5			312	0		
" "	0	10			61	0		
1,000	300	30					0	920
" "	0						0	31,200
" "	300						0	112
" "	0						0	26,100

recovered in 6 rats treated with Hetrazan for 5 days was 91, as compared with 481 which was the average number of worms recovered in the untreated animals.

Similarly the average numbers of larvae recovered from the muscles of the treated rats were much less than those recovered from the untreated animals.

Discussion and Summary. According to the results presented above, Hetrazan reduces the number of adult *Trichinella spiralis* when the drug is administered orally to rats infected with this parasite. That the number of adult worms is reduced is also shown by the fact that the larvae recovered from the musculature of the rats, treated early during infection, are less in number than those recovered from the untreated rats.

Hetrazan, when administered orally to man is relatively non-toxic.[1] The amount of drug required to kill the microfilariae of *W. bancrofti* is well under the lethal dose for rats (one mg per kg in man as compared with an LD_{50} of 285 mg per kg in rats). Hewitt *et al.*[2] have reported that the amount of drug necessary to kill the cotton rat filarid is from 10 to 25 mg per kg which has to be given constantly for a period of 2 to 4 weeks. However, the amount of drug necessary to act on the filarid worm of man is considerably less (one mg per kg 3 times daily for 5 days). Although the same species of *Trichinella* is involved in infection of man and rat it may be that, as in the case of the filarial infections, the amount of the drug necessary to overcome the infection in man is less than the amount needed for the treatment of rats.

Hetrazan acts on other intestinal nematodes particularly on *Ascaris* of man and dog.[3] It is suggestive that this drug may be useful mixed with the animal feed in order to treat infections with *Ascaris* and intestinal trichinae.

Work is in progress to determine the effect of Hetrazan on the migratory and muscle stages of *T. spiralis*.

[2] Hewitt, R., Kushner, S., Stewart, H., White, D. E., Wallace, W., and SubbaRow, Y., in press.

[3] Unpublished data.

3. No significant relief of inhibition was obtained with either nicotinic acid or tryptophane.

4. *Staphylococcus aureus* and *Mycobacterium tuberculosis* (607) were not inhibited by indoleacetic acid at the concentrations studied.

5. Fairly consistent stimulation at 0.1 mg/ml levels of indoleacetic acid were obtained with *L. arabinosus* and *L. casei* in the presence of limiting concentrations of tryptophane.

6. The significance of these results in relation to possible metabolite-antagonist relationship between indoleacetic acid and tryptophane or nicotinic acid is discussed.

Treatment of Experimental Intestinal Trichinosis with 1-Diethylcarbamyl-4-Methylpiperazine Hydrochloride (Hetrazan*).

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The drug 1-Diethylcarbamyl-4-methylpiperazine Hydrochloride (Hetrazan), when administered orally, causes the rapid disappearance of the microfilariae of *Wuchereria bancrofti* from the blood of infected individuals.[1] The marked effect of this drug on the circulating forms of *W. bancrofti* suggested its use in other parasitic infections in which treatment should be directed against such migrating forms. In view of the need for a drug in trichinosis which would destroy the migrating larvae as well as the intestinal forms, Hetrazan was tested on white rats infected with *Trichinella spiralis*. The effect of this drug on the intestinal trichinae is reported below.

Methods and Results. White rats weighing from 150 to 175 g were fed by stomach tube with 1,000 to 1,300 infective trichinae larvae. The larvae were obtained from rat muscle digested in a pepsin-hydrochloric acid mixture. Twenty-four hours after feeding the animals were started on Hetrazan. The drug

was given on the basis of 200 mg per kg weight 3 times daily by stomach tube, for periods of 5 to 10 days. For the recovery of adult trichinae the treated animals and suitable controls were killed 24 hours after the last dose of the drug was administered. The small and large intestine were removed, slit open and cut into small pieces about 1 cm long. The sliced intestine was put in a Baermann apparatus with normal salt solution heated to 37°C. The adult worms settled rapidly to the tip of the funnel and were removed one hour after the apparatus was set up. The number of worms recovered from the intestines was determined directly by counting.

For the recovery of the muscle stages the rats were killed on or about the 30th day after feeding infective larvae. The rat muscles were ground and digested in a pepsin-hydrochloric acid mixture. The larvae were recovered from the mixture and their number estimated by counting aliquot portions of the total suspension.

The average number of adult worms recovered from the rats treated with Hetrazan was considerably less than the number recovered from the untreated animals (Table I). Thus the average number of worms

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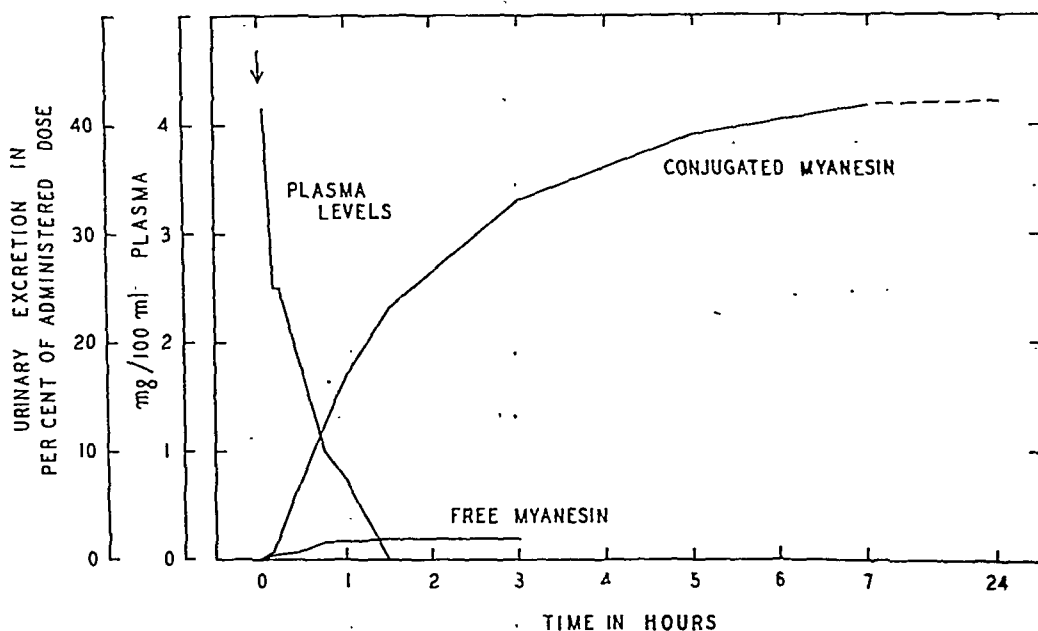[1] Santiago, D., Oliver-González, J., and Hewitt, R. I., in press.

FIG. 1.
Plasma Levels and Urinary Excretion of Myanesin.
The arrow in the upper left hand corner represents the time of the intravenous injection of myanesin, 50 mg per kg. Values plotted correspond to those of Dog No. 4 in Table II.

(Autoclaving similar solutions for 2 and 4 hours at 115-120° C did not increase the recovery of myanesin.) After removal from the bath, 5 ml of water are added to the flask, and well mixed. Five ml are then withdrawn and placed in a small separatory funnel, and the procedure as outlined under plasma followed. The 2 ml of aqueous solution eventually analyzed represent 0.5 ml of urine; consequently the final concentration value must be multiplied by 4 to yield a value reading in mg per 100 ml for the 2 ml analyzed. Note: Analysis of urine for free myanesin must be accomplished within a few hours after the sample is obtained, since spontaneous hydrolysis of the conjugated fraction gradually increases the values of free myanesin.

Analysis. Two ml of the aqueous solution of the plasma or urine extract are placed in a test tube. To each tube is added 1 ml of conc. nitric acid (sp. gr. *1.42*). The tubes are then placed in a boiling water bath for exactly 4 minutes. The tubes must not be inserted until the temperature of the water

is at least 98°C. Less than 3 minutes in the bath gives inadequate nitration; greater than 5 minutes contributes heavily to the destruction of some of the myanesin present. At the end of 4 minutes the tubes are removed and placed immediately in a beaker of cold water. When the solutions have reached room temperature, 2 ml of 40% sodium hydroxide are slowly added to each tube, with swirling to insure complete mixture.

Ten minutes after the addition of the sodium hydroxide, the contents of each tube are transferred to a clean, dry Klett tube and read in the colorimeter. The plasma or urine reagent blanks are set to zero; readings can then be converted into concentration values directly by comparison with the standard curve.

A Klett-Summerson photoelectric colorimeter was used in our determinations. employing the No. 42 (blue) filter (400-460 millimicrons wave length transmission). Using the method outlined above, the standard curve was prepared with aqueous solutions of myanesin of known concentration, and

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Plasma Levels and Urinary Excretion of Injected Myanesin in Dogs.[*]

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The recent publications of Berger and Bradley[1,2] describing a new synthetic curarizing agent, α-β-dihydroxy-γ-(2-methylphenoxy)- propane (myanesin), and the initial clinical report by Mallinson[3] of its use as a substitute for curare in 118 cases, are of potential interest in the field of anesthesia.

The brevity of action of myanesin aroused our interest in its physiological disposition, and it was considered desirable to study blood levels and urinary excretion. This report describes a method developed for the estimation of myanesin in plasma and in urine, and experimental data of plasma levels and urinary excretion in dogs.

Principle of the Method. The method described here is dependent upon the nitration of myanesin in aqueous solution, and the development of a strong yellow-green color when made alkaline with sodium hydroxide. The yellow-green color is stable for at least 60 minutes, and its intensity is measured with the photoelectric colorimeter. The myanesin is extracted from blood plasma, or from urine, with chloroform and the chloroform evaporated to dryness. The residue is then dissolved in water and an aliquot analyzed. By acid hydrolysis of the urine before the extraction with chloroform much larger quantities of myanesin can be recovered, indicating the presence of a conjugated derivative of myanesin in urine.

Method of Analysis. Extraction. (1) Plasma.[‡] Five ml of plasma obtained from oxalated blood are placed in a small separatory funnel, and 10 ml of chloroform (technical grade) are added. After thorough shaking for 8 minutes the chloroform layer is separated from the plasma, and filtered through Whatman No. 40 filter paper. Occasionally, especially in hot weather, the plasma and the chloroform may emulsify, but rarely is it impossible to obtain 5 ml of clear chloroform filtrate. A 5 ml aliquot of the chloroform filtrate is evaporated just to dryness on a water bath heated to 90°C, being careful not to overheat the residue. Two and five-tenths ml of distilled water are added to the residue, and gently warmed to aid solution of the myanesin. After a few minutes, 2 ml of this aqueous solution are placed in a test tube 16.5 x 150 mm, to be submitted to analysis. This 2 ml aqueous fraction represents the extracted myanesin from 2 ml of plasma.

(2) Urine.[‡] (a) Extraction of free myanesin. Four and five-tenths ml of urine are placed in a small separatory funnel and 1.5 ml of buffer having a pH of approximately 7.3 (prepared by adding 0.1 M citric acid to 0.2 M Na_2HPO_4 in the proportion of 2:13) are added. Twelve ml of chloroform are added, and the procedure as outlined for plasma followed, except that an aliquot of 8 ml of chloroform filtrate is evaporated to dryness, and the residue is dissolved in 3 ml of water; the 2 ml of aqueous solution of urine extract placed in the test tube for analysis represent 2 ml of urine.

(b) Extraction of free and conjugated myanesin. In a small flask 2.5 ml of urine are placed, and 2.5 ml of conc. HCl (sp. gr. 1.178-1.188) are added. The contents are then heated on a boiling water bath for 10 minutes.

1 Berger, F. M., and Bradley, W., *Brit. J. Pharmacol.*, 1946, **1**, 265.
2 Berger, F. M., and Bradley, W., *Lancet*, 1947, **1**, 97.
3 Mallinson, F. B., *Lancet*, 1947, **1**, 98.

‡ Reagent blanks of myanesin-free plasma and urine must be run concomitantly, since small amounts of plasma and urine constituents are extracted and nitrated.

TABLE II.
Plasma Levels and Urinary Secretion.

		Time in min.								
Dog	Solution	5	10	15	25	40	60	90	180	300
1	Plasma	4.90	2.85	2.42	2.35	0.85	0.70	0.0	—	—
3	,,	2.63	2.10	—	1.22	1.50	0.88	0.0	0.0	—
4	,,	4.16	2.50	2.50	1.00	1.00	0.73	00	—	1.93

Mg myanesin per 100 ml plasma

		Time in min.								
Dog	Solution	10	20	25	40	45	60	90	120	180
1	Urine	—	0.010	—	0.054	—	0.084	0.107	0.119	0.133
3	,,	0.132	—	0.275	0.433	1.57	0.527	0.527	—	0.527
4	,,	0.19	—	0.32	—	—	1.62	1.93	—	1.93

Free myanesin in % of administered dose

		Time in min.								
Dog	Solution	10	25	40	60	90	180	240	300	420	24 hrs
2†	Urine	1.51	5.31	10.41	14.92	23.02	27.06	33.7	30.98	38.1	40.2
3	,,	0.62	6.2	12.4	16.3	22.4	32.6	—	39.0	31.18	31.18
4†	,,	—	—	—	—	—	28.2	—	—	41.9	42.0
5*†	,,	—	—	—	—	—	—	—	—	—	34.0

Combined myanesin in % of administered dose

* Given 75 mg per kg myanesin.
† Foley bag catheter not employed here.

given myanesin vomited within 2 minutes after the injection. No free myanesin could be detected in the 90-minute plasma sample of any of the dogs. Conjugated myanesin, though probably present in plasma, could not be detected quantitatively by the above method of acid hydrolysis.

Urinary Output. (Table II). Output of free myanesin was followed in 3 dogs by means of periodic urine samples withdrawn through an indwelling Foley bag catheter. To insure urine blanks that give low readings one must obtain dilute urine for the determinations. In this experimental work on dogs it was found that water diuresis, the water being administered by stomach tube the evening before and every 2 or 3 hours throughout the day of the experiment in quantities of 50 to 75 ml per kg of body weight, greatly enhanced the accuracy of the determinations, since an output of light straw-colored urine could thus be insured.

The rate of excretion of free myanesin was found to parallel the concentration of myanesin in the plasma. However, only from 0.133 to 1.93% of the administered dose was detected as free myanesin in the urine; and the excretion of free myanesin was complete when the plasma level reached zero. (Fig. 1). The concentrations of free myanesin obtained in urine are so low, that the failure of urine collected from rabbits, after large doses of the drug, to have any paralytic effect on mice[1] is readily understood.

Output of conjugated myanesin was followed in 4 dogs; in 2 samples were collected by means of the Foley bag catheter, and in two by recovering the urine from the cage as it was normally passed. From Fig. 1 and Table II it can be seen that the excretion of conjugated myanesin persists for 24 hours or more in some dogs, although the greatest quantity is excreted within 3 or 4 hours. From 32 to 42% of the administered dose is excreted in conjugated form in 24 hours. However, the water diuresis necessary for this procedure may have given results somewhat at variance from normal animals.

Conjugation Product. Myanesin bears a chemical resemblance to propylene glycol

TABLE I.
Recovery of Myanesin Added to Plasma and to Urine.

Sample	Myanesin added (γ/ml)	Myanesin recovered (γ/ml)	% recovery
5 ml dog plasma	25	24.0	96.0
	50	48.5	97.0
	75	69.0	92.0
5 ml human plasma	10	10.0	100.0
	100	86.8	86.8
		Avg	94.4 ± 2.3%
4.5 ml dog urine*	7.5	5.1	68.0
	10	7.4	74.0
	50	42.3	84.6
	75	61.0	81.3
4.5 ml human urine	10	9.0	90.0
	25	24.0	96.0
	75	67.0	89.3
		Avg	83.3 ± 4.0%
2.5 ml dog urine†	10	12.4	124.0
	25	27.6	110.4
	50	47.8	95.6
	50	52.0	104.0
	50	38.0	76.0
	50	38.1	76.2
	50	34.5	69.0
	75	70.1	93.5
	100	95.6	95.6
		Avg	93.8 ± 5.9%

* Analyzed directly for free myanesin.
† Submitted to procedure for hydrolysis before analysis.

plotted to read in mg per 100 ml. The reading of the reagent blank was adjusted to zero on the colorimeter. A linear relationship exists between concentrations of myanesin and colorimeter readings for values from 0.5 to 10.0 mg per 100 ml (5 to 100 gamma per ml) of solution analyzed. Periodic checks on aqueous solutions of myanesin, ranging from 0.001 to 1.0% in concentration, showed that such solutions undergo no deterioration for at least 30 days, if stored in a refrigerator.

Experimental. Recovery Data. Table I shows recovery data obtained from analysis of dog and human plasma and urine to which known amounts of free myanesin have been added. Recovery from plasma yields values 94.4 ± 2.3% of the theoretical concentration values; from urine by analysis for free myanesin 83.3 ± 4.0% of the theoretical values; from urine containing free myanesin but submitted to treatment with hot hydrochloric acid 93.8 ± 5.9% of the known values. No conjugated myanesin was available to test hydrolysis and recovery by this

method. Probably the increase in % recovery from urine after acid treatment was due to greater extractability. However, the method outlined gave maximal values with urine from dogs previously given myanesin.

Plasma Levels. (Table II). Four dogs after absence of dehydration was assured were given myanesin intravenously in doses of 50 mg per kg and one dog, 75 mg per kg, employing 2-3% supersaturated aqueous solutions.[1] Three were followed as to plasma levels by periodically withdrawing blood samples. Fig. 1 shows a typical plasma level curve (Dog No. 4 of Table II). The sharp decay of the curve of free myanesin correlates well with the response of the animal. Immediately following the injection of 50 mg per kg, the dogs exhibited a flaccid paralysis, affecting predominently the hind legs. No reflexes or responses to painful stimuli could be elicited. Within 2 minutes after the injection all dogs were able to stand, though unsteadiness and muscular weakness persisted for about 20 minutes. Three of the 5 dogs

TABLE I.
Effect of Hemorrhage on Intestinal Absorption of Chloride in the Presence of Sulfate.

	Control* %	Experimental† %	Difference %	"p"‡
Absorption of chloride	69	81	12	.16
,, ,, sulfate	24	30	6	.15
,, ,, fluid	34	44	10	.11
Final conc. of chloride	0.187	0.138	0.049	.15
,, ,, ,, sulfate	1.17	1.22	0.05	.20

* 14 animals.
† 15   ,,
‡ Significant when "p" (according to Fisher) is 0.05 or less.

The experimental animals were etherized and blood equal to 3% of the body weight was withdrawn from the cannulated femoral artery. The artery was ligated and the incision closed. Four hours were allowed for recovery from the ether and adjustment of the circulatory mechanism. During this interim, water was allowed *ad libitum*. The control animals were subjected to the same procedure without, of course, being bled.

Under sodium barbital anesthesia (300 mg/kg intravenously) the small intestine was exposed and practically the entire ileum used for a loop. The same length of loop was used in the control and the bled animal. It was washed out with isotonic glucose solution and filled with a fluid composed of equal parts of isotonic sodium chloride and isotonic sodium sulfate solutions. The resulting mixture contained Na 0.647%; Cl 0.055%, and SO_4 1.278%.

Forty minutes later the fluid remaining in the intestine was removed and the amount carefully measured. An aliquot portion was digested with concentrated HCl and the amount of sulfate determined gravimetrically by precipitation with barium chloride. A second aliquot was used to determine the chloride as sodium chloride by the Van Slyke modification of the Volhard method.

Results and Discussion. The absorption of the chloride ion in the presence of the sulfate was not significantly increased by hemorrhage, although there was a trend in that direction. Since a hemi-isotonic sodium chloride solution was used, it was necessary for absorption to take place against a distinct gradient. Perhaps this explains why hemorrhage did not significantly accelerate the absorption of

chloride as it had when isotonic solutions of sodium chloride were used.[3] As previously noted, Goldschmidt and Dayton[1] observed that chlorides were absorbed more rapidly from the colon when a sulfate was present. It has not been shown, to our knowledge, however, that this effect obtains in the small intestine.

Less absorption of chloride and fluid was noted in the control animals. As in our previous absorption studies a fair degree of parallelism was observed between the absorption of the chloride ion and fluid.

At the end of the 40-minute period both the control and experimental animals showed definite chloride impoverishment. This was more pronounced, on the average, in the latter group but, presumably because of the wide individual variation in the several animals, the difference was not statistically significant. In both groups the sulfates showed a definite increase in concentration, because more water than sulfate was absorbed. This increase was not unexpected in the control dogs. In our laboratory it has recently[4] also been shown that a pronounced hemorrhage does not significantly affect the absorption of magnesium sulfate from the small intestine.

Summary. The absorption of chloride, sulfate and fluid from a mixture of equal parts of isotonic sodium chloride and isotonic sodium sulfate solution was studied in dogs which had suffered a hemorrhage of 3% of their body weight.

The percentages of absorption in control

4 Van Liere, E. J., Northup, D. W., Stickney, J. C., and Richard, R. E., PROC. SOC. EXP. BIOL. AND MED., 1947, 64, 62.

which has been shown by Neubauer[4] and confirmed by Fellows et al.,[5] to stimulate an increase in the excretion of glucuronic acid (conjugation?). However, glycerol itself and ethylene glycol did not produce such an increase when administered gastrically or hypodermically in rabbits.[5]

Preliminary observations would seem to indicate that myanesin is conjugated with glucuronic acid, at least in part. Neuberg and Schewket[6] have devised a method for the extraction and identification of conjugated glucuronic acid which they claim to be specific. By employing this method of extraction un-

[4] Neubauer, O., Arch. exp. Path. u. Pharmakol., 1901, 46, 133.

[5] Fellows, J. K., Luduena, F. P., and Hanslik, P. J., J. Pharmacol. and Exp. Therap., 1947, 89, 210.

[6] Neuberg, C., and Schewket, O., Biochem. Z., 1912, 44, 502.

equivocal reactions were obtained with naphthoresorcinol,[6,7] although inconsistent reactions were obtained with orcinol,[6,7] on urine excreted after the administration of myanesin, whereas similarly treated control urines gave only the weakly positive reactions expected of normal urine.

Summary. A colorimetric method has been presented for the determination of myanesin in plasma and in urine. The rapid decay curve of myanesin in dog plasma explains the brevity of its pharmacological action. In the dog, from 0.1 to 2.0% of the administered dose is excreted as free myanesin; from 32 to 42% of the administered dose is excreted as conjugated myanesin in 24 hours, possibly as the glucuronide.

[7] Hawk, P. B., and Bergheim, O., Practical Physiological Chemistry, Philadelphia, P. Blakiston's Son & Co., Inc., 11th edition, 1937, pp. 68, 658.

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The Null Effect of Hemorrhage on Intestinal Absorption of Chloride in the Presence of Sulfate.*

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Goldschmidt and Dayton[1] showed that sodium sulfate increases the absorption of sodium chloride in the large intestine of dogs. Burns and Visscher[2] later demonstrated that the presence of sodium sulfate and certain other anions of the lyotropic series caused the chloride ion, at a concentration below that in the blood plasma, to leave the small intestine and enter the blood against its diffusion gradient.

Van Liere, Northup, and Sleeth[3] noted that

[1] Goldschmidt, S., and Dayton, A. B., Am. J. Physiol., 1919, 48, 459.

[2] Burns, H. S., and Visscher, M. B., Am. J. Physiol., 1934, 110, 490.

isotonic sodium chloride solution was absorbed significantly faster from the intestine of dogs which had sustained a severe hemorrhage (3.2% of their body weight). Since hemorrhage thus simulated the effect of the sulfate ion, it was of interest to study their combined effect.

Methods. Dogs, selected in pairs as nearly alike in weight and age as possible, were fasted 24 hours previous to the experiment. One served as a control and the other was subjected to hemorrhage. Fifteen experiments were performed on a total of 29 animals (one control died).

[3] Van Liere, E. J., Northup, D. W., and Sleeth, C. K., Am. J. Physiol., 1938, 124, 102.

TABLE I.

Acceleration of Coagulation by Iodinated and Untreated Trypsin. Each tube contained 0.5 ml of plasma and 0.1 ml of 1% CaCl$_2$·2H$_2$O plus other additions as indicated.

No.	NaCl 0.9% ml	Non-iodinated trypsin 0.1% ml	Iodinated trypsin			Avg coagulation time, sec	% of normal coagulation time
			1.6 × 10⁻³ m Eq iodine per mg trypsin	1.8 × 10⁻³ m Eq iodine per mg trypsin	2.0 × 10⁻³ m Eq iodine per mg trypsin		
1	0.1	0	0	0	0	755	100.0
	0	0.1	0	0	0	68	9.0
	0	0	0.1 ml of 0.3%	0	0	75	9.9
2	0.1	0	0	0	0	221	100.0
	0	0.1	0	0	0	37	16.7
	0	0	0.1 ml of 0.1%	0	0	47	21.2
	0	0	0.1 ml of 0.3%	0	0	35	15.8
3	0.1	0	0	0	0	338	100.0
	0	0.1	0	0	0	47	13.9
	0	0	0	0.1 ml of 0.3%	0	50	14.8
4	0.1	0	0	0	0	475	100.0
	0	0.1	0	0	0	54	11.4
	0	0	0	0.1 ml of 0.3%	0	65	13.7
5	0.1	0	0	0	0	358	100.0
	0	0.1	0	0	0	54	15.1
	0	0	0	0	0.1 ml of 0.1%	95	26.5
6	0.1	0	0	0	0	430	100.0
	0	0.1	0	0	0	65	15.1
	0	0	0	0	0.1 ml of 0.1%	116	27.0
	0	0	0	0	0.1 ml of 0.3%	76	17.7
7	0.1	0	0	0	0	130	100.0
	0	0.1	0	0	0	41	31.5
	0	0	0	0	0.1 ml of 0.3%	44	34.0
	0	0	0	0	0.1 ml of 0.5%	40	30.9
8	0.1	0	0	0	0	127	100.0
	0	0.1	0	0	0	41	32.2
	0	0	0	0	0.1 ml of 0.1%	48	37.8
	0	0	0	0	0.1 ml of 0.3%	44	34.6
	0	0	0	0	0.1 ml of 0.5%	42	33.0

time was considerably less and the accelerating influence of untreated or iodinated trypsin was less apparent. Plasma and the diluent or the enzyme were placed in tubes having inside measurements of 5 x 55 mm and allowed to come to 38°C in a water bath. Calcium chloride was then added, a stopper quickly inserted and the tubes were slowly and uniformly rocked back and forth under the surface of the 38° bath until the air bubble in the tube became stationary and a clot was observed. With these small tubes it was found desirable to begin timing the coagulation when the tubes were first inverted with the calcium chloride present.

The crude trypsin was iodinated under conditions described earlier.[14]

Results. The data of Table I indicate that iodinated trypsin, as well as the untreated enzyme, considerably hastens the coagulation of recalcified plasma. The difference in the degree of acceleration by the untreated and by the iodinated enzyme is similar to the difference in their proteolytic activities described earlier.[14] Approximately the same degree of acceleration by iodinated trypsin can be accomplished by employing 3 to 5 times as much material. However, like the rate of proteolysis, acceleration of coagulation is not directly proportional to the concentration of the enzyme and appreciable acceleration results when iodinated trypsin is used

and experimental dogs were, respectively, 69 and 81 of the chloride; 24 and 30 of the sulfate; and 34 and 44 of the fluid.

In both control and experimental animals the sulfates showed a definite increase in concentration at the end of the experimental period, the chlorides a definite impoverishment. However, the differences between the control and the experimntal animals were not statistically significant.

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Influence of Iodinated Trypsin on Blood Coagulation.*

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It has been known for some time that the addition of trypsin accelerates the coagulation of normal blood[1-4] and the blood of hemophilic individuals.[5,6] Intravenously administered trypsin is, however, quite toxic as pointed out by Eagle and Harris.[4] The similarity between the symptomatology resulting from the injection of the enzyme and anaphylactic reactions was observed by Rocha e Silva[7] and these observations have been extended by Dragstedt and his associates.[8-12] Shock resulting from the injection of a relatively small amount of trypsin is indicated by a marked fall in blood pressure.

Tagnon[13] slowly injected trypsin intravenously in hemophilic patients and observed a significant shortening of the coagulation time; however, he has advised extreme caution since the dose most effective on clotting time is very near to that which is quite toxic.

In an earlier report[14] the author pointed out that the iodination of trypsin serves to decrease greatly its hypotensive effect while its proteolytic activity is decreased only about one tenth as much. Relatively large amounts of the iodinated enzyme do not cause the profound fall in blood pressure observed with the same amount of untreated trypsin even with rapid injection.

The present report deals with the influence of iodinated trypsin on the rate of blood coagulation *in vitro*.

Methods. Freshly drawn dog blood was mixed with 3.3 mg of sodium citrate per ml of blood. The plasma was separated after centrifuging for 2 hours at 3200 r.p.m. With short periods of centrifugation the clotting

1 Douglas, S. R., and Colebrook, L., *Lancet*, 1916, **2**, 180.

2 Heard, W. N., *J. Physiol.*, 1916, **51**, 294.

3 Waldschmidt-Leitz, E., Stadler, P., and Steigerwaldt, F., *Naturwissenschaften*, 1928, **16**, 1027.

4 Eagle, H., and Harris, T. N., *J. Gen. Physiol.*, 1936-37, **20**, 543.

5 Tyson, T. L., and West, R., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 494.

6 Ferguson, J. H., and Erickson, B. N., *Am. J. Physiol.*, 1939, **126**, 661.

7 Rocha e Silva, M., *Arquiv. Inst. Biol.*, Sao Paulo, 1939, **10**, 93.

8 Ramirez de Arellano, M., Lawton, A. H., and Dragstedt, C. A., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 360.

9 Rocha e Silva, M., and Dragstedt, C. A., *J. Pharm. and Exp. Therap.*, 1941, **72**, 36.

10 Dragstedt, C. A., and Rocha e Silva, M., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 420.

11 Rocha e Silva, M., and Dragstedt, C. A., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 152.

12 Wells, J. A., Morris, H. C., and Dragstedt, C. A., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 209.

13 Tagnon, H. J., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 45.

14 Bowman, D. E., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 408.

reaches zero throughout the system for a portion of each respiratory cycle during which period settlement by gravity is possible. However, it is generally accepted in standard texts[2] that respiratory movements do little other than ventilate the respiratory passages down to the finer subdivisions of the bronchioles (Henderson *et al.*[3]). A gas finds its way to the depth of the alveoli only by molecular diffusion. Since nebulized substances are much larger than molecules of a gas, the laws of diffusion do not apply. Nebulized particles, therefore, are transported only when there is an active movement of a column of air, at which time the forces of inertia and impingement causing deposition of such particles are greatest.

An additional factor preventing penetration and retention is the return of some of the particles in the inspired air to the outside atmosphere with expiration. Brown[4] has shown that retention of dust particles is inversely proportional to the respiratory rate for rates below 20 per minute, thus, the slower the respiration the greater the retention. He also concluded that the percentage retention was not affected by volume per respiration, vital capacity or relative humidity of inspired air.

It is often stated that a 5 micron particle will float airborne to terminal air passages. This statement is based on the fact that the terminal bronchioles are 300-400 microns in diameter, and thus it is assumed that a 5 micron particle should have no difficulty in making the passage. This assumption is open to criticism.

Van Wijk and Patterson[5] studied the proportion of particles of different sizes removed from dust-laden air by breathing. They found

that 25% of 0.2 micron particles are removed, 80% of 2 micron particles are removed, and 95% of 5 micron particles are removed. They further state that the larger particles are deposited in the larger air passages, thus, the smaller the particle, the less the retention. Bryson and co-workers[6] made photomicrographs of penicillin aerosol reporting the average particle diameter to be 0.54 microns with a range of 0.24 to 1.18 microns.

It would appear from these purely theoretical considerations that large concentrations of aerosol mists will not penetrate deeply into the respiratory tree because

1. The forces of inertia and impingement causing deposition are greatest in the larger branches;
2. The moving air column does not penetrate to the alveoli;
3. The mist will not diffuse as will a gas;
4. There is a return to the outside of some of the mist; and
5. The larger particles which are most likely to be retained are most likely to be deposited in the larger bronchioles.

The literature reveals only one experimental study which separates clearly the factor of pharyngeal absorption from actual penetration of mist. Krueger and co-workers[7] confined an adult monkey in an atmosphere of dilute India ink for 60 minutes. On gross examination, following autopsy, carbon was found in large amounts in the nasal passages, larynx and stomach, in smaller amounts in the trachea and bronchi. Microscopic sections showed that some inspired material had penetrated into the alveolar spaces. The following experiments were undertaken to investigate the apparent lack of agreement between the theoretical considerations and the experimental result.

Methods. The depth of penetration and magnitude of concentration of various nebulized substances in the respiratory tree was determined experimentally in 26 animals (2

[2] a. Bachmann, G., and Bliss, A. R., Jr., *Essentials of Physiology and Pharmacodynamics*, Blakiston Co., Philadelphia, 1940; b. Best, C. H., and Taylor, B. T., *The Physiological Basis of Medical Practice*, 3rd ed., p. 512, Williams and Wilkins, Baltimore, 1939.

[3] Henderson, Y., Chillingworth, F. P., and Whitney, J. L., *Am. J. Physiol.*, 1915, **38**, 1.

[4] Brown, C. E., *J. Indust. Hyg.*, 1931, **13**, 285; *ibid.*, 1931, **13**, 293.

[5] Van Wijk, A. M., *J. Indust. Hyg.*, 1940, **22**, 31.

[6] Bryson, V., Sansome, E., and Laskin, S., *Science*, 1944, **100**, 33.

[7] Personnel of Naval Laboratory Research Unit No. 1, Command of Krueger, A. P., and Lyons, W. R., *Am. J. Med. Sci.*, 1944, **207**, 40.

in amounts equal to the optimum amount of the untreated material.

Further study of the numerous factors to be considered in the use of iodinated trypsin *in vivo* is in progress.

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Summary. The coagulation of recalcified plasma, *in vitro,* is hastened considerably by trypsin which has been iodinated sufficiently to greatly decrease its hypotensive effect.

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Depth of Penetration of Nebulized Substances in the Respiratory Tree.[*]

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(Introduced by E. Ogden.)

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To effect a local concentration of penicillin in the pulmonary system, some physicians have been administering nebulized penicillin in the inspired air.

Administering penicillin mist by the usual method of inserting a spray into the oral pharynx uses two routes of administration simultaneously. The mist deposited on the pharyngeal mucosa is absorbed, diluted by the blood stream and distributed throughout the body; also some of the mist may follow the column of inspired air down the respiratory passages to effect a local surface action. Intramuscular injection of penicillin produces higher blood concentrations than is possible by mucosal absorption of the relatively small dosage used in nebulizers. Thus, the only advantage of nebulization is a possible local action on the surfaces of the respiratory passages.

The purpose of this study was to determine the magnitude of concentration and the depth of penetration down the respiratory passages, of a nebulized substance administered with inspired air.

Liquids, such as Lipiodol, pass rapidly to the terminal bronchioles and alveoli. It is known from unpublished war research that aerosol clouds in which the mass median diameter is carefully controlled will penetrate

in small concentrations to the alveoli. Dusts, such as silica, over long periods of exposure may penetrate deeply. This study is not concerned with such phenomena, but only with conditions similar to those of aerosol therapeusis, *i.e.*, mixture, with commercial nebulizers, of mist with inspired air in the oral pharynx, and durations of exposure of 15-60 minutes of eupneic breathing.

Nebulized particles in inspired air tend to be deposited against the walls of the respiratory system by settlement due to gravity and by changes in the direction of air flow. The particles tend to be retained in the system by impingement on moist and adhesive surfaces. The deposition due to changes in direction of air flow increases with the velocity of air flow and turbulence, whereas settlement, due to gravity, is only effective where the velocity is low.

Hatch[1] states that maximum air velocities occur in the nasal passages. The mean velocity of the air column in the bronchioles is reduced to 1/1000, and at the entrance of the alveoli is further reduced to 1/100,000 of nasal velocity. Thus the greatest deposition of nebulized particles should be expected in the upper respiratory tree. The air velocity

[1] Hatch, T. F., Behavior of Microscopic Particles in the Air and in the Respiratory System, p. 102, in *Aerobiology*, Moulton, F. R., editor, publication of the A.A.A.S., No. 17, Washington, D.C., 1942.

larger quantities of carbon and iron were deposited. No particulate matter was demonstrated in the smaller bronchioles or alveoli.

5. Because a satisfactory microscopic picture—for a comparison control—of the deposition of carbon, dye or iron in the alveoli had not yet been produced, an attempt was made to force particulate matter into the alveoli. The lungs and trachea were removed in one block from 2 dogs and 2 rats and were artificially inflated by the nebulizer. A free flow of mist was insured by cutting the tip from one of the lower lobes. The lungs were inflated and deflated beyond physiological limits for a period of 15 minutes. Microscopic sections indicated only a minute amount of foreign material in the bronchi. None of the alveoli was seen to contain foreign material. Even those alveoli adjacent to the free flow of mist at the cut tip were free of particulate matter.

6. The breathing of one dog was stimulated by an intermittent mixture of CO_2 with the inspired air. Dilute India ink mist was introduced into the oral pharynx for a period of 30 minutes. The gross and microscopic distribution was essentially the same as in dogs during the eupneic breathing. No carbon was distributed in the smaller bronchioles or alveoli.

7. Abramson[8] has suggested that 25% glycerol be added to the carbon suspension to lower vapor pressure and thus make a more stable mist. Two dogs were exposed to a glycerol mist containing carbon for 30-minute periods. There were tracheobronchial depositions of carbon in larger quantities than when aqueous suspensions were used. In a dependent lobe of one dog there was a sharply localized area 2 cm in diameter in which carbon was deposited in the alveoli. However, in this dog there was an excess of free flowing fluid in the oral pharynx and the dog coughed and "gurgled" several times during the procedure. It is believed that this localized alveolar deposition was due to aspiration and flow rather than to inspiration of nebulized mist.

Discussion. A careful search of the literature fails to bring to light any actual experimental confirmation of Krueger's experiment[7] in which the factor of pharyngeal absorption is clearly separated from the factor of inspiratory penetration.

Using nebulized sulfonamide solutions, Mutch[9] found that only 7 to 14 percent of the drug leaving the nebulizer reached the blood stream. Later, working with nebulized penicillin, Mutch and Rowell[10] found that only 25% of the mist was absorbed into the blood stream. Neither of these studies determined what proportion of the blood concentration was due to pharyngeal absorption. We have been able to demonstrate only a minimal penetration to the larger bronchi, and a questionable penetration of nebulized substance into the deeper respiratory passages.

Although penicillin aerosol may be adequate for effecting some degree of pharyngeal absorption, it does not yield adequate surface action on the respiratory passages. It is the impression of the authors that the concentration of the substance on the surfaces of the trachea, bronchi and bronchioles would not be sufficient for therapeutic effectiveness.

Summary. 1. An attempt has been made to determine the depth of penetration, and the amount of deposition in the respiratory system of nebulized dilute India ink, aqueous gentian violet solution and aqueous ferric ammonium sulfate solution in 2 mice, 11 rats, 2 rabbits and 11 dogs.

2. The substances were found to be deposited in decreasing amounts in the oropharynx, larynx, bifurcation of the trachea, the bronchi and bronchioles of the animals. The amounts of deposited substances in the bronchi and bronchioles were so small as to make microscopic detection difficult. In no case was any nebulized substance found to have reached the alveoli.

3. There is no evidence to support the theory that pulmonary lesions can be treated by topical application with commercial nebulizers.

8 Abramson, H. A., *Ann. Allergy*, 1946, 4, 440.

9 Mutch, N., *Lancet*, 1944, 2, 775.

10 Mutch, N., and Rowell, R. E., *Lancet*, 1945, 1, 650.

TABLE I.

1	2	3	4	5	6
Angle of bend in glass tube	No bend	one 30° angle	one 90° angle	one 120° angle	two 20° angles
% of mist removed from air column	defined as 0%	30-35%	75-80%	90-95%	50%

mice, 11 rats, 2 rabbits and 11 dogs). A commercial nebulizer ("glaseptic"—Parke-Davis & Co.) connected to a half-liter pressure bottle was used to obtain a fine mist of diluted India ink (1:1 or 1:5 aqueous dilution), gentian violet (1%-5% aqueous solution) or ferric ammonium sulfate (1%-5% aqueous solution). It was found that a hand bulb gave a more sustained mist with smaller droplets than did oxygen at 8 to 10 liters per minute; so the former was used exclusively after the first 4 animals studied.

The size of the nebulized droplets sprayed from the pressure bottle was directly observed by comparison with red cell smears to include particles as small as 0.5 microns with 80% being 1 to 3 microns in diameter.

The animals were lightly anesthetized with pentobarbital sodium (Nembutal). The mists were introduced to the respiratory tree by a glass tube placed in the oral-pharynx, or in some animals by a tracheal cannula placed in the same axis as the trachea. The rate and depth of respirations were noted. The animals were exposed to the mists for periods of from 15 to 60 minutes. They were immediately sacrificed and gross and microscopic observations were made.

Preliminary experiments on the action of suspensions passing through a system of branching glass tubes demonstrated the removal of particulate matter from the air column at the points where the direction of the air column changed. The amount of nebulized suspension of dilute India ink passing through a straight tube was accepted as a measure of 0% removal (Table I). As the angle of bend in the tube increased, and as the number of bends in the tube increased, there was an increase in the removal of particulate matter from the air column. The carbon was deposited largely on the walls opposing flow at the bends in the tubing.

Results. 1. The first group of one rat and

2 mice was not anesthetized nor was an oral cannula used. The animals were placed in an atmosphere of a mist of 50% India ink for 60 minutes. Carbon particles were found in the nasopharynx and stomach of each animal. No carbon was seen grossly or microscopically in the larynx, trachea, bronchi, bronchioles, or alveoli.

2. Because the nasal passages appeared to be removing most of carbon from the inspired air in the first group, a second group of 7 rats and 2 rabbits, lightly anesthetized, was exposed to India ink, gentian violet or ferric ammonium sulfate solutions for periods of from 15 to 30 minutes. The mist was introduced to the inspired air by an oral cannula. The nose was occluded and the tongue pulled forward to insure mouth breathing.

Particles were seen grossly to be present in rather large quantities in the oral pharynx and larynx of all animals. Below this point the nebulized substances were noted in small quantities, mostly at the point of branching of the bronchi. Microscopic sections indicated no evidence of foreign material in the alveoli. Only one cell out of every one or two thousand lining the bronchi showed any evidence of foreign material.

3. Because of the negative results in rats and rabbits, a third group of 4 dogs was exposed to India ink suspensions (1:5 aqueous dilution) and ferric ammonium sulfate (1% aqueous solutions) with exposures of from 30 to 60 minutes. The results were essentially the same as with the second group of animals except that in two animals there were minute particles in the larger bronchioles which may have been either artifact or inspired material.

4. In order to by-pass the screening of the inspired air by the pharynx and larynx, the mist was directly introduced through a tracheal cannula into a rat and 2 dogs. This fourth group of animals showed tracheobronchial distribution similar to the other groups, but
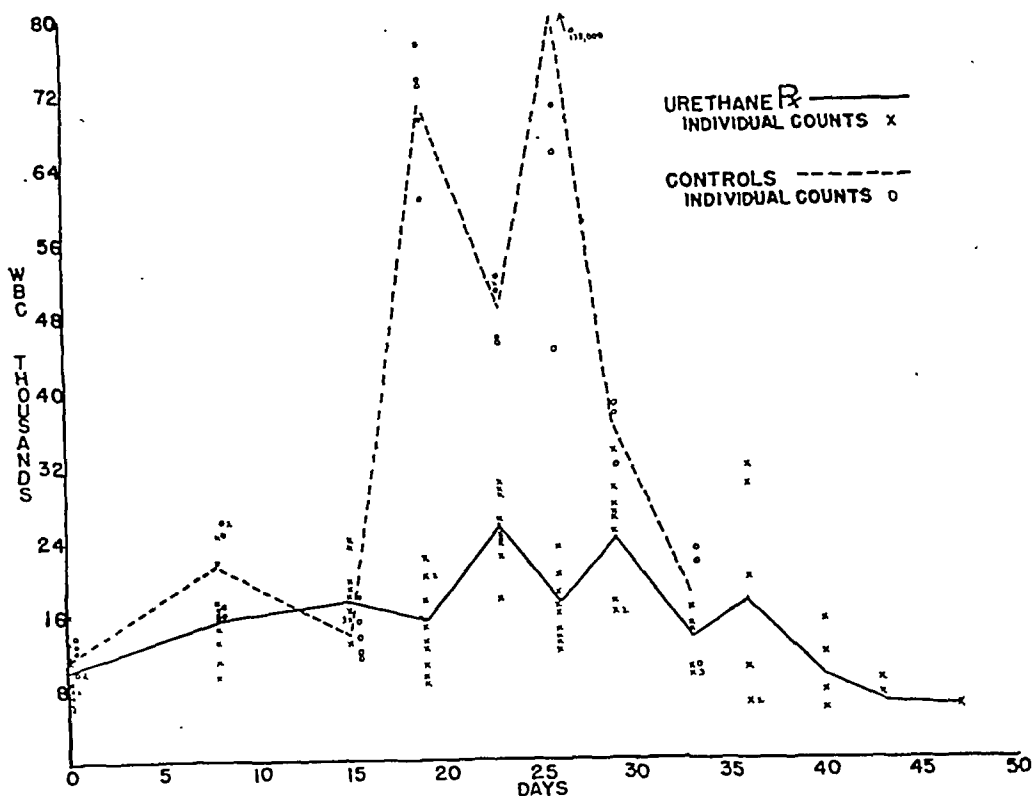
FIG. 1.

Average and individual white blood cell counts of the urethane-treated group and the control group are shown. Only a very slight rise above normal occurred in the urethane group.

In Fig. 2 the averages of the differential counts are plotted. The change in counts of the animals which were given cell suspension and not treated with urethane was entirely characteristic of the transplanted disease. Evidence of leukemia was manifest in the peripheral blood on the 19th day, at which time primitive blast cells were first seen. The leukemia was at its peak on the 26th day and all the animals were dead by the 34th day. The drop in white blood cell count shortly before death is usually seen. The treatment with urethane had a striking effect on the course of the transplanted leukemia. The total and differential counts showed no change from the normal until the 23rd day at which time there was a slight rise above normal and a few blast cells appeared. The slight rise was maintained until the 40th day at which time the total counts fell to within normal limits.

A small percentage of blasts, however, persisted until the death of the last animal on the 48th day. The urethane had the effect of delaying the appearance and greatly diminishing the number of white blood cells in the peripheral blood.

The histologic changes in the control group not treated with urethane were typical of the transplanted disease. The infiltration of the organs lagged behind the development of leukemia as manifested by changes in the peripheral blood. The 2 animals which survived only 22 and 24 days showed only moderate infiltration. The 3 which survived 31, 31, and 34 days showed maximal infiltration. The animals treated with urethane also developed infiltration in the organs but this occurred later and to a less marked degree than in the untreated animals. One mouse that survived 27 days showed no infiltration.

16060

Effect of Urethane on Transplanted Leukemia of Ak Mice.*

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Haddow and Sexton,[1] investigating the effect of urethanes on human malignant tumors, reported that they observed a fall in the leucocyte counts. Prompted by this Paterson, Haddow, Thomas, and Watkinson[2] treated 32 cases of human leukemia, 19 myeloid and 13 lymphatic, with urethane, (ethylcarbamate). In both types of leukemia they observed a decrease in the total white blood cell count with a return towards normal of the differential pattern. This was accompanied by decrease in the size of the spleen and lymph nodes and a rise in the hemoglobin level. They considered the effects to be comparable to those obtained with deep x-ray therapy. There was no evidence that permanent benefit resulted from the therapy. Engstrom, Kirschbaum, and Mixer[3] tested the effect of urethane on the transplanted myelogenous leukemia in F strain mice. They found that just sub-lethal doses were effective in reducing the white blood cell counts to normal but did not eradicate the infiltration of the tissues. Smaller doses had a less marked effect on the counts. Kirschbaum and Lu[4] demonstrated later that a single anesthetic dose of urethane decreased the number of mitotic figures in marrow myeloid cells and reduced the total number of circulating white cells, especially myeloblasts, in transplanted myeloid leukemia of F strain mice.

Mice of the Ak strain have a very high incidence of spontaneous leukemia. About 75% develop the disease after the age of 6 months. The intravenous injection of leukemic cells from diseased older animals produces progressive leukemia in 100% of younger animals. The leukemic tissue is cut into small pieces with scissors and immersed in Tyrode's solution. The larger particles are allowed to settle leaving a suspension of cells in the supernatant fluid. The concentration of cells in the suspension is determined by counting in a hemacytometer. Dilution is usually necessary until the required concentration is reached. The injection into the tail vein of about 40,000 cells suspended in 0.10-0.15 cc of solution produces leukemia in all of the young animals in about 2½ weeks and death occurs in 3 to 5 weeks.

Twenty Ak animals† less than 5 months old were selected and divided into 3 groups. Five received the cell suspension only; 10 received cell suspension and were treated with urethane; and 5 were treated with urethane only. It had been determined previously that the maximum dose tolerated over a 5-week period by mice weighing 25-30 g was 7.5 mg injected subcutaneously twice daily. This dosage was used. White blood counts and differential counts were done once weekly for 2 weeks and then twice weekly. At autopsy sections of the liver, spleen, and bone marrow were taken for histologic study.

The white blood cell counts of the animals which were treated with urethane and were not injected with cell suspension did not show any variation from the normal. The average and individual total white blood cell counts of the other two groups are shown in Fig. 1.

1 Haddow, A., and Sexton, W. A., *Nature* (London), 1946, **157**, 500.

2 Paterson, E., Thomas, I. A., Haddow, A., and Watkinson, J. M., *Lancet*, 1946, **250**, 677.

3 Engstrom, R. M., Kirschbaum, A., and Mixer, H. W., *Science*, 1947, **105**, 255.

4 Kirschbaum, A., and Lu, C. S., Proc. Soc. Exp. Biol. and Med., 1947, **65**, 62.

16061

Alloxan Diabetes in Hypophysectomized Rats.[*]

C. Cabell Bailey, Philip M. LeCompte, Orville T. Bailey, and Clifford C. Franseen. (Introduced by E. P. Joslin.)

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Since diabetes can be produced in certain animals by the injection of alloxan[1,2,3] or by the injection of crude anterior pituitary extract,[4-7] this investigation was undertaken to determine whether alloxan produces its diabetogenic effect through the pituitary gland.

For many years an association between the pituitary and diabetes mellitus has been suggested by (1) the high incidence of diabetes and glycosuria among acromegalics,[8] (2) the production of experimental diabetic animals by the injection of crude anterior pituitary extract,[4-7] (3) the marked hypersensitivity to insulin exhibited by hypophysectomized animals,[9] (4) the amelioration of the diabetes in depancreatized dogs or toads following hypophysectomy,[10,11] (5) the suggestion of pituitary overactivity in children[12a] in the few months preceding the onset of diabetes as evidenced by rapid dental and osseous development beyond that expected for their age.

Although both alloxan and crude anterior pituitary extract (A P E) produce islet cell destruction and diabetes when injected into dogs, there are some striking differences in their action, the most outstanding of which are as follows:

(1) The injection of APE produces diabetes only in the dog and in the partially depancreatized rat or cat,[13] whereas alloxan has already been shown to produce diabetes in the dog, rat, cat, rabbit, pigeon, monkey, sheep and turtle.[12a]

(2) Hyperglycemia is imperative for the production of diabetes with APE.[13] The use of insulin, phlorhizin or a low carbohydrate diet which prevents hyperglycemia also prevents the development of diabetes. Alloxan however produces islet cell necrosis and diabetes irrespective of hyperglycemia.[14]

(3) Alloxan produces islet cell destruction and diabetes within 24 hours[15] after injection of a single dose, whereas APE must be injected daily for several days or weeks.[4]

(4) Pancreatic islet lesions produced with a single diabetogenic dose of alloxan show degenerative changes from the start with destruction of all or nearly all of the beta cells within 24 hours.[15] No similar rapid islet destruction follows APE but rather a gradual degranulation with the development of extensive hydropic degeneration of the beta cells.[16]

The production of diabetes by the injection

[*] We are indebted to the American Cyanamid Company for a research grant.

[1] Bailey, C. C., and Bailey, O. T., *J. A. M. A.*, 1943, **122**, 1165.

[2] Goldner, M. G., and Gomori, G., *Endocrinology*, 1943, **33**, 297.

[3] Dunn, J. S., and McLetchie, N. G. B., *Lancet*, 1943, **2**, 384.

[4] Young, F. G., *Lancet*, 1937, **2**, 372.

[5] Houssay, B. A., Biasotti, A., and Rietti, C. T., *C. R. Soc. de Biol.*, 1932, **111**, 479.

[6] Evans, H. M., Meyer, K., Simpson, M. E., and Reichert, F. L., Proc. Soc. Exp. Biol. and Med., 1931-32, **29**, 857.

[7] Baumann, E. J., and Marine, D., Proc. Soc. Exp. Biol. and Med., 1931-32, **29**, 1220.

[8] Davidoff, L. M., *Endocrinology*, 1926, **10**, 461.

[9] Houssay, B. A., and Magenta, M. A., *Rev. Assn. Med. Argent.*, 1924, **37**, 389.

[10] Houssay, B. A., and Biasotti, A., *C. R. Soc. de Biol.*, 1930, **104**, 407.

[11] Houssay, B. A., and Biasotti, A., *C. R. Soc. de Biol.*, 1930, **105**, 121.

[12] Joslin, E. P., Root, H. F., White, P., Marble, A., and Bailey, C. C., *The Treatment of Diabetes Mellitus*, 8th Edition, Philadelphia, Lea and Febiger, 1946, (a) p. 743, (b) p. 178.

[13] Lukens, F. D. W., *Yale J. Biol. and Med.*, 1944, **16**, 301.

[14] Goldner, M. G., and Gomori, G., Proc. Soc. Exp. Biol. and Med., 1944, **55**, 73.

[15] Bailey, O. T., Bailey, C. C., and Hagan, W. H., *Am. J. Med. Sci.*, 1944, **208**, 450.

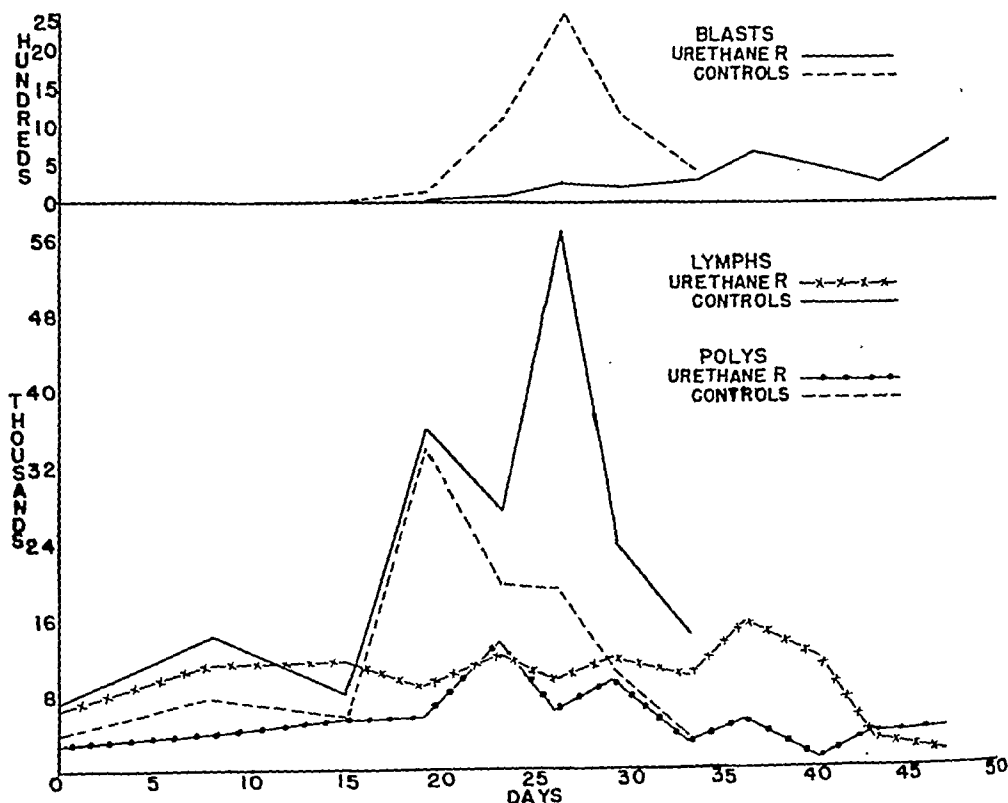[16] Duff, G. L., *Am. J. Med. Sci.*, 1945, **210**, 381.

FIG. 2.
Average differential counts of the urethane-treated group and the control group are shown.
Primitive blast cells appeared later and in smaller numbers in the urethane group.

Another that survived 29 days showed minimal infiltration in the spleen and one small focus of leukemic cells in the liver. In the animals that survived 32 to 48 days there was moderate infiltration which was much less extensive than that present in the untreated animals surviving 31 to 34 days. There was a general tendency to weight loss which was not extreme in any one group and was not more or less marked in any one group.

The urethane treatment increased the survival time of the animals injected with cell suspension. The average survival time of those not treated with urethane was 28.4 days, of those treated with urethane 36.0 days. This is statistically significant. P equals 0.05.[5] Of the 5 animals which were treated with ure-

[5] Fischer, R. A., *Statistical Methods for Research Workers*, Oliver and Boyd, Edinburgh, 1934.

thane and not injected with cells one died on the 19th day and one on the 41st. The other 3 were killed on the 50th day. It is probable that the dose of urethane was toxic and was approaching a lethal dose.

Conclusions. The administration of urethane to Ak mice with transplanted leukemia caused a delay in the appearance of immature cells in the peripheral blood and prevented a marked increase in total white blood cell count and numbers of immature blast cells. Infiltration of organs occurred later and to a less marked degree. The average survival time of the animals was increased and this increase was statistically significant. In order to secure these effects a dosage of urethane was used which was just sub-lethal. It is probable that the drug itself was partly responsible for the death of the treated animals.

blood sugars below 200 mg %. Three of the rats later responded to a larger dose of alloxan with a blood sugar over 200 mg %, but all 3 again had a subsequent blood sugar less than 150 mg %. In other words, the blood sugar levels showed wide fluctuations and a striking tendency to revert to normal.

Group D—*Animals Apparently Resistant to Alloxan.* The 4 animals in this group were given from 2 to 5 injections of alloxan, 2 of them receiving a maximum dose of 200 mg per kg and the other 2 a maximum dose of 300 mg per kg without developing hyperglycemia. It is noteworthy that one animal showed apparently complete loss of beta cells from the islets of Langerhans and another showed an acute degenerative change in the beta cells probably due to the last injection of alloxan.

Pathological Findings. In 2 animals (one from Group B, one from Group D), the histological preparations were unsatisfactory owing to postmortem change. The islets of Langerhans in the remaining 17 rats showed loss of beta cells in varying degree, with apparent complete loss of beta cells in the majority, the islets being composed of alpha cells staining pink with the Gomori chromealum hematoxylin method. A few surviving beta cells were present in some islets of 2 rats in Group A, one in Group C, and one in Group D. No definite necrosis or other lesion of organs such as liver, kidney, and adrenal was noted. The expected findings in the hypophysectomized animals—*e.g.*, atrophy of gonads, adrenals, and thyroid—were also present.

Discussion. Hypophysectomized rats fail to grow and their fur remains fine and soft. Their weight tends to remain constant and they show an abnormal response to both insulin and to adrenalin. These animals show marked hypersensitivity to insulin and respond to about one-tenth the dose of insulin required to act in normal animals.[19] Adrenalin produces less hyperglycemia than it does in the intact animal. Since hypophysectomized rats show a marked tendency to hypoglycemia

and since the administration of alloxan is followed by a transitory hypoglycemia, especial care must be taken to prevent fatal hypoglycemia in hypophysectomized rats given alloxan. For this reason, glucose water was made available.

Perhaps the most striking feature of the experimental results is the large number of animals showing diabetes which was either transitory or fluctuating. These included not only the rats in Group C (transitory group), but also the 3 rats in Group B (long duration), all of which showed one or more normal blood sugars during their course. In other words, even in the permanently diabetic hypophysectomized animal, the blood sugar showed a definite tendency to revert to normal.

In unoperated animals made diabetic with alloxan, a certain number may be expected to exhibit a transitory course, but the fluctuations were not so striking as in the present series. In a previous series of 50 control rats given a dose of 150 mg in this laboratory, the results were classified as follows:

	%
Permanent diabetes	58
Transitory diabetes	12
Resistant to 150 mg	22
Died before first blood sugar	8

In this control group the rats exhibiting permanent diabetes showed sustained high blood sugar levels, without reversion to normal, while those classed as transitory showed high blood sugar levels for a week or so, the subsequent ones being within the normal range. In other words, the striking fluctuations seen in the hypophysectomized animals were not present in the controls.

It is also noteworthy, as remarked above, that all the animals in which good histological preparations were obtained showed destruction, to a greater or less degree, of the beta cells in the islets of Langerhans; *i.e.* the anatomical lesion was present regardless of the apparently mild course of the diabetes.

The phenomenon of amelioration of diabetes following hypophysectomy is, of course, not new, being well known in the "Houssay animal", where hypophysectomy is followed

19 Russell, J. A., *Physiol. Rev.*, 1938, **18**, 2.

of repeated small doses of alloxan produces some degranulation and hydropic degeneration in the beta cells but not to a degree comparable to that following the administration of APE.[15]

In view of these findings, it seemed appropriate to investigate whether the presence of the pituitary is essential for the production of alloxan diabetes. This report deals with hypophysectomized animals injected with alloxan.

Materials and Methods. White rats of the Hisaw strain, obtained from the Biological Laboratory of Harvard University, were used. They were maintained on Purina Fox-Chow and water. After hypophysectomy glucose water was also made available at all times. (See Discussion.)

Hypophysectomy was done by one of us (C.C.F.) using a parapharyngeal approach through the base of the skull, the technique of which has been described in detail elsewhere.[17]

Approximately 2 weeks after operation, a control micro blood sugar determination (Folin-Malmros method) was done on blood obtained by cutting off the tip of the rat's tail. Blood samples were obtained near the middle of the day throughout the experiments and no attempt was made to withhold food. (See Discussion.) A freshly prepared 5% solution of alloxan (Eastman Kodak Company) was then administered subcutaneously in an initial dose of 150 mg per kg in 15 rats and 100 mg per kg in 4 rats. When no hyperglycemia occurred, the initial dose was repeated or the dose was increased by 25 or 50 mg per kg. Animals were considered diabetic when the blood sugar exceeded 200 mg % 24 or more hours after the injection of alloxan.

In evaluating the completeness of the hypophysectomy the chief clinical criterion used was failure to gain in weight. At autopsy a careful search was made for remnants of the pituitary and, if any were found, the animal was omitted from the series.

In most instances the animals were killed with ether, and autopsied immediately, the fresh tissues being fixed usually in Bouin's and Helly's fluid. In most cases sections were made of all the important organs. The pancreas was examined in all the rats included in the final series. Gomori's chrome-alum hematoxylin stain[18] proved to be most satisfactory for differentiating the beta and alpha cells in the islets of Langerhans.

Results. The final series consisted of nineteen rats. These were divided into groups on the basis of their clinical behavior, as follows:

Group A—Diabetes; animals sacrificed soon after onset	5 rats
Group B—Diabetes; animals allowed to survive for months	3 "
Group C—Transitory diabetes	7 "
Group D—Apparently resistant to alloxan in doses given	4 "

Group A—*Diabetes; Animals Sacrificed Soon After Onset.* This group comprises 5 rats sacrificed after being diabetic for periods varying from 2 to 28 days. Four of these animals had blood sugar levels over 400 mg %, after a single dose of 150 mg alloxan per kg. The fifth animal received doses of 100, 125, and 150 mg per kg at approximately weekly intervals without obvious effect but developed hyperglycemia after 175 mg per kg was given. The incidence of diabetes in this group does not appear to differ from that in normal rats injected with alloxan. (See Discussion.)

Group B—*Diabetes; Animals Allowed to Survive for Months.* The 3 rats in this group remained diabetic for relatively long periods, one for 2½ months, and 2 for 8½ months. One of these required a dose of 300 mg per kg before diabetes was definitely established. All 3 animals showed some fluctuation in blood sugar levels, all having occasional blood sugar values below 200 mg %. This point is commented upon in the discussion below. One animal developed cataracts in both eyes.

Group C—*Transitory Diabetes.* Six of the 7 animals in this group showed a single high blood sugar a few days following the first injection of alloxan and then one or more

17 Franseen, C. C., Brues, A. M., and Richards, R. L., *Endocrinology*, 1938, **23**, 292.

18 Gomori, G., *Am. J. Path.*, 1941, **17**, 395.

. 16062

Effect of Anoxic Anoxia on Bile Secretion in the Rat.

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Studies on the effect of anoxic anoxia on the absorption of fat from the alimentary tract of rats have shown that the amount of fat absorbed by animals subjected to partial pressure of oxygen of 63 mm and 53 mm Hg (8.35 and 7.03% oxygen, respectively) was significantly less than for control animals.[1] This cannot be explained on the basis of a prolonged gastric emptying time since it was found that rats fed corn oil showed an initial acceleration of the emptying of the stomach on exposure to diminished oxygen tension.[2]

Glikson and Rubel[3,4] reported that exposure of dogs with a biliary fistula to pressures equivalent to altitudes of 6,000 and 8,000 m (9.78 and 7.68% oxygen, respectively) resulted, as a rule, in a decrease in the volume of bile secreted with an increase in the content of organic solids and the viscosity. To a lesser degree and less constantly there was an increase in the bile acid content. They attributed the changes to oxygen starvation of the hepatic cells. Schnedorf and Orr[5] found that increasing degrees of anoxemia produced by inhalation of 15, 10, and 5% oxygen in nitrogen resulted in a marked and progressive decrease below normal in the flow of bile in nembutalized dogs.

Since it is generally recognized that bile salts play an important role in the digestion and absorption of fat, the decreased rate of fat absorption observed in rats subjected to reduced barometric pressure might reasonably result from a diminished flow of bile. It was considered of interest, therefore, to investigate the effect of anoxic anoxia on the secretion of bile salts in the rat.

Methods. Young adult albino rats were found to be the most suitable for the experiments. Using the bile fistula technique of Harrington, Greaves, and Schmidt,[6] the bile duct was anastomosed to the vas deferens by means of a stainless steel cannula about the size of a No. 24 hypodermic needle. Thus the bile was completely excluded from the intestinal tract and excreted in the urine instead. Six rats were successfully operated by this technique and remained in apparent health for a period of 3 to 5 months. The low-fat diet recommended by Greaves and Schmidt[7] for bile fistula rats was fed, with the exception that meat meal supplemented with 0.25% cod liver oil concentrate was substituted for fish meal which was unavailable at the time. After the rats had recovered from the operative procedure, they were exposed to simulated high altitude for 4-hour periods in individual metabolism cages. At the end of each period the volume of urine was measured and analyzed for its content of bile salts. Exposure to altitude was carried out not more often than once a week to avoid the possibility of acclimatization. Alternating with exposures to altitude control experiments were performed. In the early experiments the rats were subjected to a simulated altitude of 28,000 ft. (53 mm Hg oxygen tension). This resulted in the death of two animals, consequently in the subsequent ex-

1 MacLachlan, P. L., and Thacker, C. W., *Am. J. Physiol.*, 1945, 143, 391.

2 MacLachlan, P. L., Proc. Soc. Exp. Biol. and Med., 1946, 63, 147.

3 Glikson, E. B., and Rubel, V. M., *Arch. sci. biol.* (U.S.S.R.), 1940, 58, 76.

4 Glikson, E. B., and Rubel, V. M., *Bull. biol. med. exptl. U.R.S.S.*, 1940, 9, 334.

5 Schnedorf, J. G., and Orr, T. G., *Am. J. Dig. Dis.*, 1941, 8, 356.

6 Harrington, F. G., Greaves, J. D., and Schmidt, C. L. A., Proc. Soc. Exp. Biol. and Med., 1936, 34, 611.

7 Greaves, J. D., and Schmidt, C. L. A., *J. Biol. Chem.*, 1933, 102, 101.

by improvement in the diabetes produced by pancreatectomy. The results of the present experiments are, therefore, not surprising and it would seem that there is no more evidence that alloxan acts through the pituitary than there is that pancreatectomy exerts its effect in this way.

The general question of the relation of the pituitary to carbohydrate metabolism is well discussed by Russell,[19] Houssay,[20] and Lukens.[21]

A preliminary report on the production of alloxan diabetes in hypophysectomized rats was published from this laboratory in 1945.[22] As far as we are aware, the only other reports on alloxan diabetes in hypophysectomized animals are those of Kirschbaum et al.,[23] of Duff and Starr,[24] and of Gaarenstroom.[25]

Kirschbaum, Wells and Molander[23] found that hypophysectomized rats injected with alloxan developed severe hypoglycemic convulsions but no experiments were carried beyond 6 hours and no histological sections were reported.

Duff[16] describes unpublished observations

by Duff and Starr in which hypophysectomized rats were given alloxan and some survived the initial hypoglycemic stage. Duff states that "although histologic studies showed destruction of the islets of Langerhans quite as extensive as in intact animals, there was no evidence of diabetes during the periods of survival. The blood sugar remained at more or less normal levels, but there were irregular fluctuations somewhat above and below the normal limits."

Gaarenstroom[25-27] reported that extirpation of the hypophysis in animals already made diabetic with alloxan led to a marked decrease or even total disappearance of glycosuria and a fall in blood sugar, if the animals were fasted. After administration of sugar the blood sugar rose again to high levels.

Conclusions. 1. Diabetes was produced by the injection of alloxan in 15 of 19 hypophysectomized rats. 2. The diabetes so produced tended to be either transitory or fluctuating in severity and it is suggested that this phenomenon represents the "Houssay effect" seen in depancreatized hypophysectomized dogs. 3. Extensive loss of beta cells in the islets of Langerhans was noted in all animals in which satisfactory histological preparations were obtained. The presence of hypophysis is therefore not necessary for the production of what appears to be the essential lesion in alloxan diabetes.

20 Houssay, B. A., *Essays in Biology in Honor of Herbert M. Evans,* Univ. of Calif. Press, 1943.

21 Lukens, F. D. W., *Am. J. Med. Sci.,* 1946, **212,** 229.

22 Bailey, C. C., Bailey, O. T., and Leech, R. S., *Bull. N. Eng. Med. Center,* 1945, **7,** 59.

23 Kirschbaum, A., Wells, L. J., and Molander, D., PROC. SOC. EXP. BIOL. AND MED., 1945, **58,** 294.

24 Duff, G. L., and Starr, H., unpublished work, cited by Duff.[16]

25 Gaarenstroom, J. H., *J. Endocrinology,* 1947, **5,** 103.

26 Gaarenstroom, J. H., and DeJongh, S. E., *Acta Brevia Neerlandica,* 1946, **14,** 28.

27 Gaarenstroom, J. H., DeJongh, S. E., and Polder, C. C., *Acta Brevia Neerlandica,* 1946, **14,** 70.

16063 P

Cross Transfusion as a Means of Determining Toxic Factors in Blood from Burned Animals.

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(With the technical assistance of Mr. Everett Hoppe.)

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Although numerous methods of transfusion of blood from one animal to another have been utilized in an effort to test for toxic products, most of them fail to utilize principles which might prevent dilution of the toxins. When testing for a toxin possibly arising from an extremity removal of blood from a vein in the neck or another extremity would obviously obtain blood in which any toxin would be greatly diluted. In efforts to test for a possible toxin in burned hind extremities of a dog, we resorted to cross transfusion in which 100 cc of blood per kg of body weight was removed
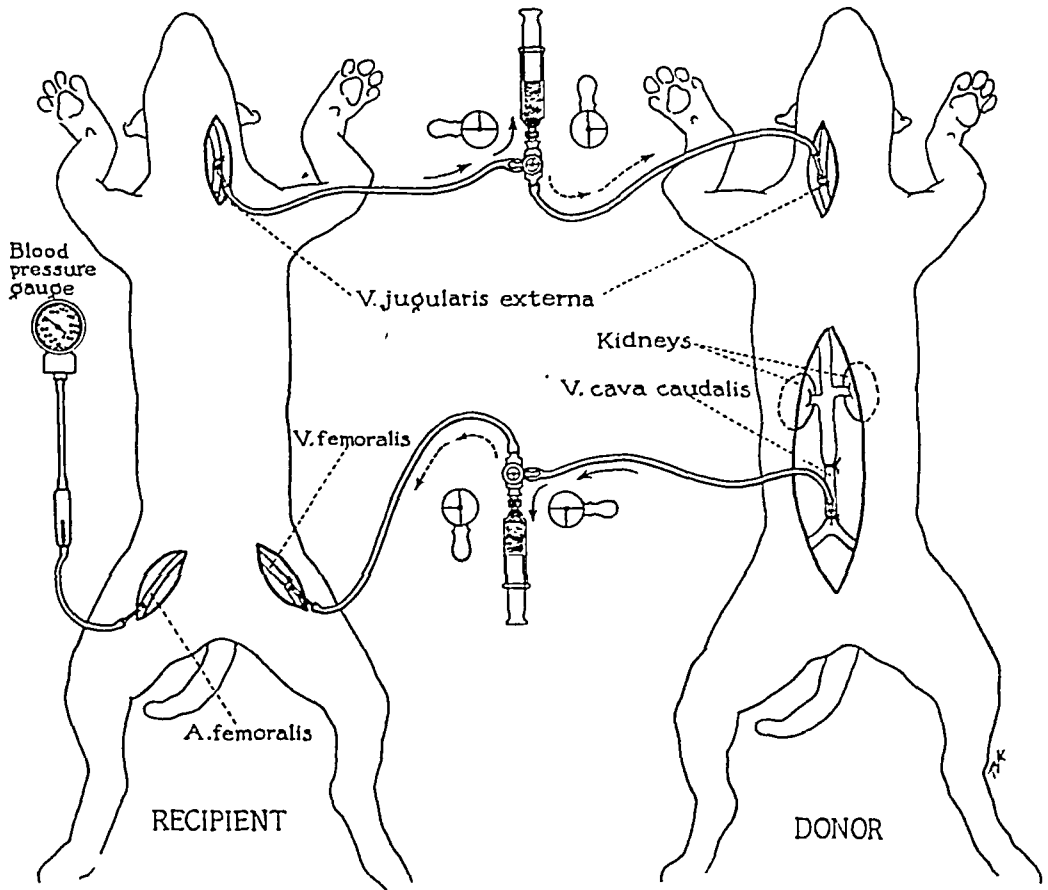
FIG. 1.

In our cross-transfusion experiments blood was removed from the vena cava of the animal (donor) burned on the lower extremities and distal torso; it was injected into the femoral vein of the normal animal (recipient). Blood was removed from the jugular vein of the normal animal and injected into the jugular vein of the burned animal.

TABLE I.
Effect of Anoxic Anoxia on Excretion of Bile Salts and Urine in 6 Bile Fistula Rats. (Average values for four-hour control and experimental periods.)

	No. of Exp.	Bile salts, mg	Urine vol., cc	Corr. coefficient bile salts/urine
Control	32	0.37	2.3	—0.4
Anoxia	32	0.34	3.9	+0.3
Std. Dev.		0.107	1.575	
P (Fisher's)		>0.2	<0.001	

periments the altitude was reduced to 24,000 ft. (63 mm Hg oxygen tension).

The urine was analyzed for its content of bile salts by the procedure of Morrison and Swalm[8] and Morrison.[9] This method was found to give satisfactory results on known solutions of cholic acid, as well as urine samples containing bile supplemented with known amounts of cholic acid.

Results and Discussion. The data, Table I, show that there was no significant difference in the amount of bile salts excreted by bile fistula rats when subjected for 4-hour periods to diminished oxygen tension as compared to control periods. In agreement with the findings of Stickney,[10] there was a statistically significant increase in the output of urine as a result of exposure to simulated altitude. Since there was no correlation between the amount of bile salts and the volume of urine excreted, the values for each were expressed in terms of the amount excreted per 4-hour period.

The range in the volume output of urine was 0.8 to 5.0 cc for the control periods, and 1.2 to 7.7 cc for the periods of exposure to altitude. Apparently the urine, irrespective of its volume, flushed out the bile which had collected in the urethra.

Although the amount of bile salts excreted during the control and experimental periods varied considerably, (0.15 to 0.62 mg and 0.16 to 0.68 mg respectively), the output over a 2- to 3-month interval did not show any noticeable decrease with increasing age of the chronic biliary fistula, as found by Boyd in dogs.[11]

Haney, Roley, and Cole,[12] as a result of studies on dogs with Thiry Vella loops, suggested that bile salts may play an important role in the normal regulation of the propulsive movements of the small intestine. Ackerman, Curl and Crandall,[13] however, working with bile fistula dogs found that in general their data did not lend support to the concept that bile salts are an important factor in the regulation of small intestine motility. Since in rats anoxic anoxia was found to be without effect on the secretion of bile salts, a changed motility of the small intestine as a result of deficient bile secretion cannot be considered of importance as far as the effect of anoxia on fat absorption is concerned.

Summary. No significant difference was found in the amount of bile salts excreted by 6 bile fistula rats when exposed for 4-hour periods to decreased oxygen tension (63 mm and 53 mm Hg) as compared to control periods. There was a statistically significant increase in the volume output of urine as a result of exposure to simulated altitude. No correlation existed between the amount of bile salts and the volume of urine excreted.

8 Morrison, L. M., and Swalm, W. A., *J. Lab. Clin. Med.*, 1940, **25**, 739.

9 Morrison, L. M., *J. Lab. Clin. Med.*, 1943, **28**, 1503.

10 Stickney, J. C., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 210.

11 Boyd, E. M., Earl, T. J., Jackson, S., Palmer, B., and Stevens, M. E. T., *Am. J. Physiol.*, 1945, **145**, 186.

12 Haney, H. F., Roley, W. C., and Cole, P. A., *Am. J. Physiol.*, 1939, **126**, 82.

13 Ackerman, R. F., Curl, H., and Crandall, L. A., Jr., *Am. J. Physiol.*, 1941, **134**, 32.

16064

Certain Mathematical Aspects of the Susceptibility of Erythrocytes to Lysis.*

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Variation in the value of the parametric constant 1/n in the von Krogh alternation formula[1] has been used by several authors[2-4] as a convenient index of the susceptibility of red cells to lysis in the presence of complement and a hemolytic antiserum. It is of interest to consider in some detail the relationship between the value of the constant and the distribution of susceptibility to lysis among individual red cells.

As ordinarily expressed, von Krogh's formula is:

$$x = k \left(\frac{y}{1-y}\right)^{\frac{1}{n}} \qquad (1)$$

where x = lysin concentration

y = fraction of cells hemolyzed

k, 1/n are constants for a given set of reagents.

The plot of y versus x (Fig. 1) is a sigmoid curve which provides an excellent fit for experimental points between the limits y = 0.1 and y = 0.9. The lack of fit beyond these limits has been attributed to the rapid rate of change of y/1—y as y approaches zero or unity.

Solving Equation 1 for y gives:

$$y = \frac{x^n}{k^n + x^n} \qquad (2)$$

and, differentiating with respect to x,

$$\frac{dy}{dx} = \frac{nk^nx^{n-1}}{(k^n + x^n)^2} \qquad (3)$$

If x is expressed in terms of 50% hemolytic units, then it may be seen from Equa-

tion 1 that, at 50% hemolysis (y = 0.5), the fraction y/1—y is unity and k = 1. This choice of the units of x allows Equation 3 to be simplified to:

$$\frac{dy}{dx} = \frac{nx^{n-1}}{(1 + x^n)^2} \qquad (4)$$

The derivative dy/dx represents the rate of change of the degree of hemolysis with respect to lysin concentration. The value of the derivative at any point x is then proportional to the number of cells that require that concentration of lysin for hemolysis. Thus the curve of dy/dx versus x will provide a graphic representation of the distribution of susceptibility among the red cells of a given population.

The shape of this curve for 3 commonly encountered values of 1/n is shown in Fig. 2. The asymmetry of the curve, which suggests that the lysin is proportionally less effective in the higher concentrations, conforms with Ponder's view,[5] based on experimental data, that the accumulated products of hemolysis have an inhibiting effect on the lysin.

The respective curves for the 3 values of 1/n disclose that there is an inverse relationship between the value of 1/n and the uniformity of a given cell population with respect to susceptibility to lysis. It is suggested that any condition which leads to a change in uniformity will lead to an inverse change in the value of 1/n. This supposition is consistent with the observation of Kent[4] that the optimal degree of cell sensitization with an homologous antiserum occurs at a minimum value of 1/n for the system, and with the disclosure of Morse[2] that 1/n tends to increase either with aging of the cells, or on the introduction of certain impurities.

* Aided by a grant from the Commonwealth Fund.

1 von Krogh, M., J. Infect. Dis., 1916, 19, 452.

2 Morse, S., Proc. Soc. Exp. Biol. and Med., 1922, 19, 17.

3 Wadsworth A., Maltaner, E., and Maltaner, F., J. Immunol., 1931, 21, 313.

4 Kent, J. F., Science, 1947, 105, 316.

5 Ponder, E., Proc. Roy. Soc., B, 1923, 95, 382.

TABLE I.

Mortality Rate, Blood Pressure, and Pulse Changes in Animals Receiving Blood from Normal Control Animals as Compared to Animals Receiving Blood from Burned Animals.

–	Receiving blood from normal animals	Receiving blood from burned animals
Mortality rate	16.6%	27.7%
Avg drop in blood pressure	19.35 mm Hg	41.83 mm Hg
Avg rise in pulse rate	12.9 beats per min	14.8 beats per min
Total No. of experiments.	30	18

over a 25-minute period from the distal vena cava of the burned animal (donor) and transfused into the jugular vein of the normal animal (recipient), as illustrated in Fig. 1. This obtained blood directly from the area of the burn which was produced by immersion of the distal portion of the animal for 30 seconds up to the third thoracic vertebra in vegetable oil heated to 120°C. All animals used in these experiments were anesthetized with 33 mg sodium pentobarbital per kg of body weight injected intraperitoneally; ether was used in a smaller series not reported herein. Blood from the 2 animals being cross-transfused was matched previously to rule out incompatibility. To facilitate transfusion, 5 mg heparin per kg of body weight was injected intravenously into the animals. The cross-transfusions were performed 48 to 96 hours after the burn, representing a time shortly before the expected death of the burned animal.

The mortality rate in 30 dogs receiving blood from the vena cava of normal animals was 16.6% compared to a rate of 27.7% in 18 dogs receiving blood from the vena cava of animals burned on the lower extremities and distal torso. (Table I) The average

drop in blood pressure of dogs receiving blood from normal dogs was 19 mm of mercury compared to a drop of 42 mm in dogs receiving blood from burned animals. There was very little difference in pulse rate in the 2 experiments. Statistical analysis of the data on blood pressure and mortally revealed the figures to be significant although the series is small.

Summary. In our experiments studying the effect of transfusion of blood from a burned animal (dog) to a normal animal we resorted to a method of cross transfusion which obtained blood from the vena cava (draining the burned area), and cross transfused large quantities (several hundred cc) to and from the normal and burned animal. The average drop in blood pressure of dogs receiving blood from normal dogs was 19 mm of mercury, compared to a drop of 42 mm in dogs receiving blood from burned animals in the same quantity and same speed of injection. The mortality rate in 30 dogs receiving blood from the vena cava of normal animals was 16.6% compared to a rate of 27.7% in 18 dogs receiving blood from the vena cava of animals burned on the lower extremities and distal torso.

Summary. An approximate curve representing the distribution of susceptibility to hemolysis among the erythrocytes of a given population is obtained by differentiation of the von Krogh equation. The von Krogh constant 1/n is shown to be an inverse measure of the uniformity of distribution.. Variations in the value of 1/n are concluded to be indicative of variations in the uniformity of the cell suspension with respect to its susceptibility to hemolysis.

16065

Bacteria and Cellular Activities. I. Effect of *Streptococcus beta-hemolyticus* on Permeability of Chicken Erythrocytes.[*]

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In order to obtain a better understanding of some of the changes which may take place in cells under the influence of bacteria and their products, a series of experiments is being undertaken. The initial studies involved changes in the permeability of erythrocytes in the presence of a hemolytic bacterium.

Ponder[1] has reviewed the older literature on this general subject. Many similar experiments have been performed since (Maizels[2]). In all of the work cited interest was centered on the actual hemolysis of the erythrocytes. In the present investigation the permeability of the cells prior to hemolysis was considered.

The following data then are of interest not only with reference to the general problems mentioned, but also emphasize the care which must be exercised to eliminate the possible effect of bacterial contamination in permeability studies of erythrocytes.

Procedure. The test organism, *Streptococcus beta-hemolyticus* was secured from the American Type Culture Collection. It was seeded on proteose No. 3 agar and incubated for 24 hours at 37.5°C. An aliquot of the inoculum removed from the medium by washing with sterile Ringer-Locke solution was added to an equal volume of heparinized chicken erythrocytes obtained by cardiac puncture. This suspension of erythrocytes and bacteria was incubated at 37.5°C. A control suspension was made up in a similar manner except that the organisms were omitted. Aliquots were removed at varying intervals of time, and the permeability of the erythrocytes to glycerol was measured by the photronic cell technique employed by one of the authors (Hunter[3]). Change in volume of the erythrocytes alters the amount of light transmitted to the photocell, hence the current induced and the "scale reading" of the galvanometer. The volumes of the erythrocytes may be routinely varied (1) by placing them in an isotonic solution of a penetrating substance (0.3 M glycerol) which ultimately results in hemolysis or (2) by placing them in a hypertonic solution of a penetrating substance in Ringer-Locke solution (0.3 M glycerol in Ringer-Locke solution), which produces a rapid shrinkage and a subsequent swelling. The light transmission is increased both by swelling and by hemolysis. In the hypertonic solution swelling alone is meas-

[1] Ponder, E., *The Mammalian Red Cell and the Properties of Haemolytic Systems*, Berlin, 1934.

[2] Maizels, M., *Quart. J. Exp. Physiol.*, 1946, 33, 183.

[3] Hunter, F. R., *J. Cell. and Comp. Physiol.*, 1936, 9, 15.

FIG. 1.

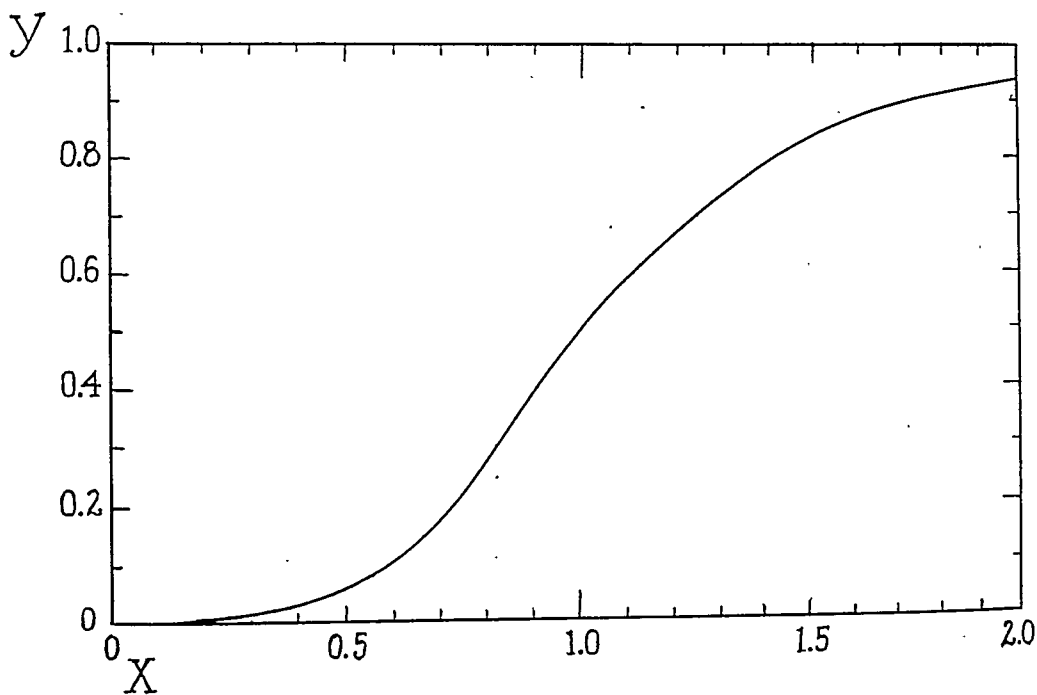$\frac{1}{m}\begin{cases}\text{———} =0.200 \\ \text{- - - -} =0.250 \\ \text{-·-·-} =0.300\end{cases}$
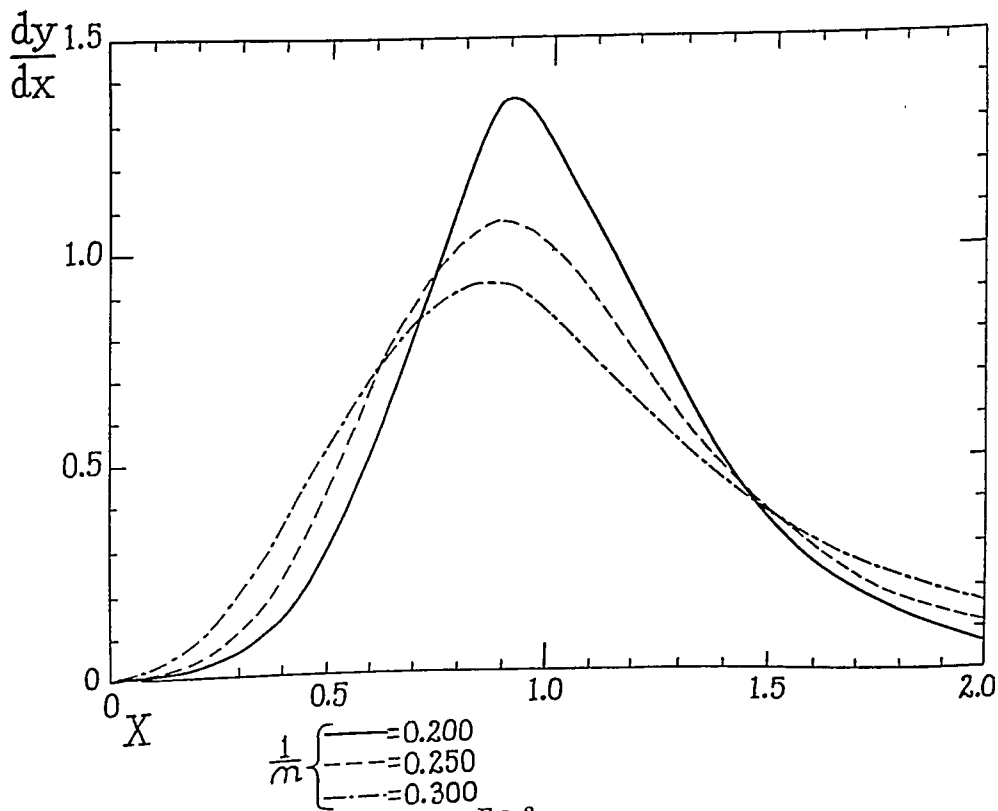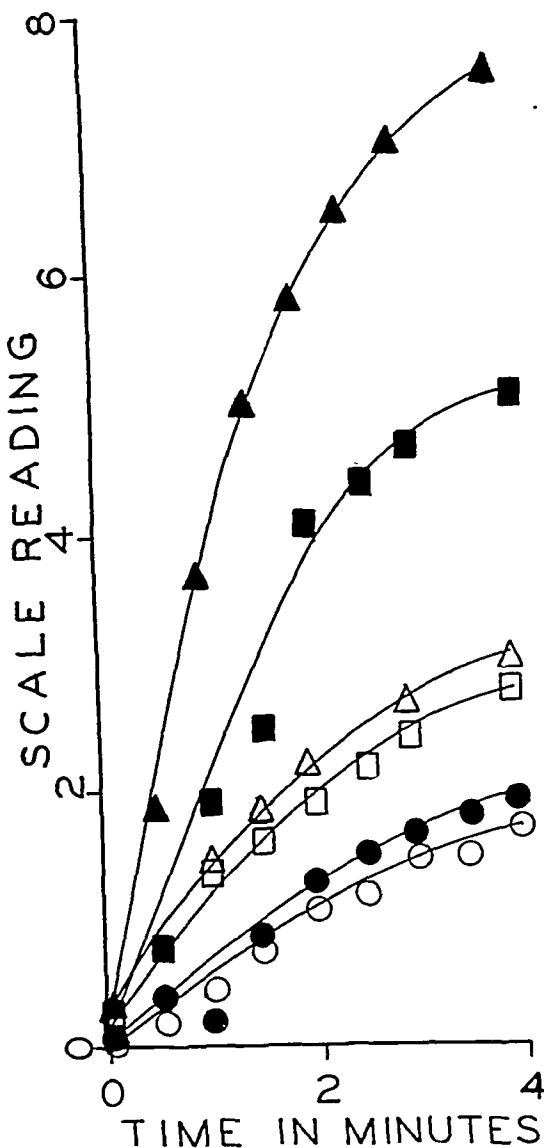
FIG. 2.

FIG. 2.

Rate of swelling of chicken erythrocytes exposed to test organism for varying times in 0.3 M glycerol in Ringer-Locke solution. ○—control, 0 hr; ●—experimental, 0 hr; □—control, 12 hr; ■—experimental, 12 hr; △—control, 24 hr; ▲—experimental, 24 hr.

ured. In the isotonic solution swelling precedes hemolysis, but since the light changes associated with hemolysis are so much greater than those due to swelling, the hemolysis curves are almost completely a consequence of the loss of hemoglobin from the cells.

Plates were poured to ascertain whether or not contamination had occurred: (1) when the blood was drawn, (2) after the erythrocytes had been centrifuged, (3) at the completion of each experiment, and (4) on the Ringer-Locke solution just before use. Only data from experiments which were free of contamination are considered. After the aliquot had been removed for the permeability studies further sterile precautions were not taken. Contamination introduced during the ten minutes or less required for the measurements would have had a negligible effect.

Results. In general exposure to bacteria for several hours increased the permeability of the cells to glycerol and decreased the time for hemolysis. (Fig. 1) To determine whether or not the change in hemolysis time was actually a consequence of a change in permeability, the rate of swelling was measured simultaneously in a hypertonic solution of 0.3 M glycerol in Ringer-Locke solution. The swelling curves are shown in Fig. 2. The decrease in the hemolysis times varies directly with the increase in the rate of swelling. As a final check fragility measurements were made. Fig. 3 shows that cells exposed to the test organism for 7 hours are not more fragile than control cells.

Discussion. The time for hemolysis and the rate of swelling of the control cells change over a period of many hours. Such a change has been noted in previous work (Hunter[4]), and might well be predicted on the basis of observations such as those of Jacobs and Parpart.[5] Preliminary experiments indicate that this change results at least in part, from volume changes of the cells. It is clear, however, that with erythrocytes the change is more marked in the presence of the test organisms.

Prior to hemolysis a gradual alteration of the cell membrane is induced by the test organism. This is indicated by the change in permeability to the non-electrolyte, glycerol. Hence, in permeability studies in which

4 Hunter, F. R., *J. Cell. and Comp. Physiol.*, 1947, 29, 301.

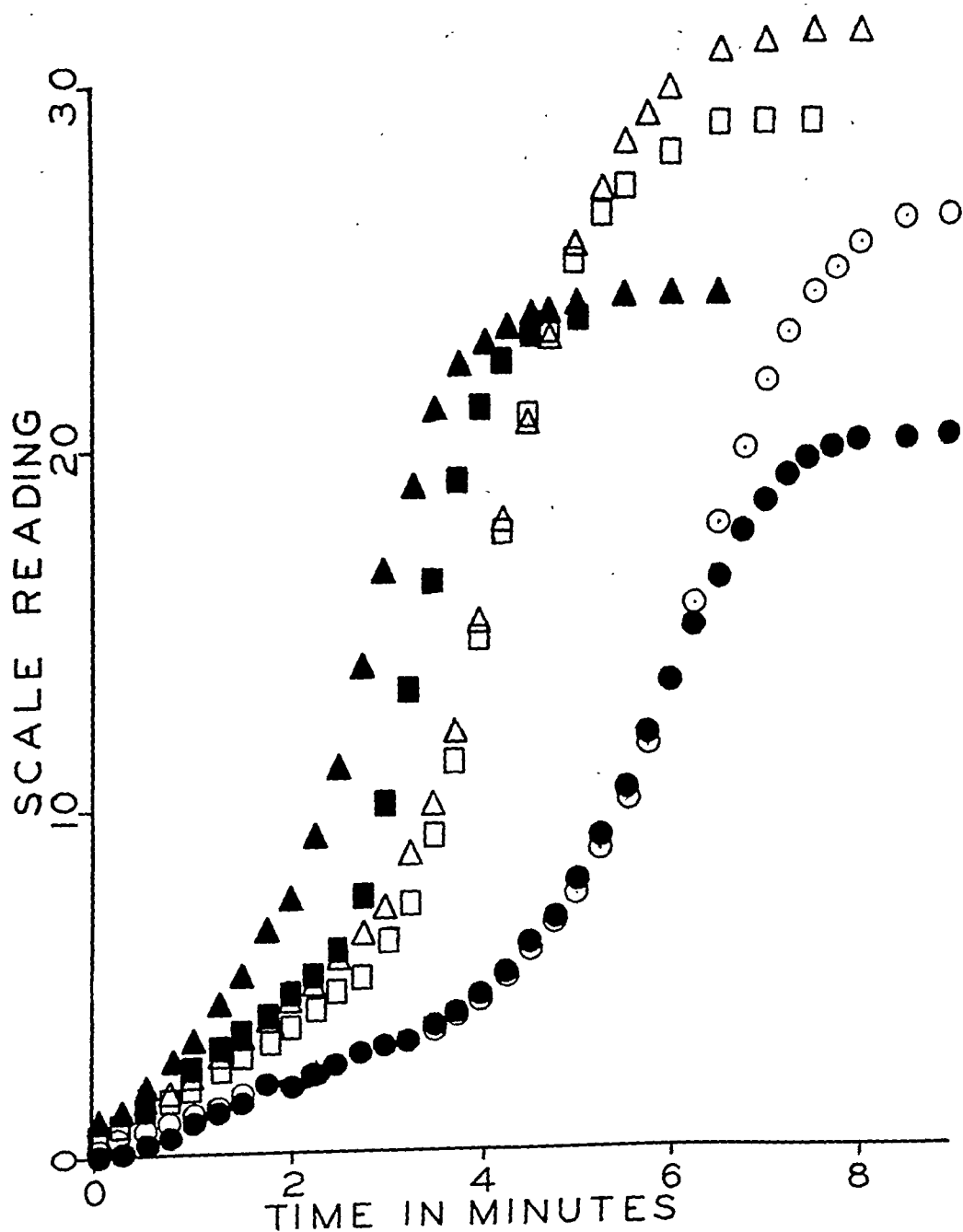5 Jacobs, M. H., and Parpart, A. K., *Biol. Bull.*, 1931, 60, 95.

FIG. 1.

Effect of time of exposure to test organism on the hemolysis of chicken erythrocytes in glycerol. ○—control, 0 hr; ●—experimental, 0 hr; □—control, 12 hr; ■—experimental, 12 hr; △—control, 24 hr; ▲—experimental, 24 hr.

16066

An Improved Device for Recording Activity of Rats.

C. C. SCOTT AND H. M. WORTH.   (Introduced by K. K. Chen.)

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Measurement of spontaneous activity of rats can be accomplished satisfactorily by the spring-suspended cage technique of Schulte and associates.[1,2] In these laboratories some

WORK ADDER

MERCURY
SWITCH

TO
COUNTER

FIG. 1.
Details of apparatus for recording movement of spring-suspended cage.

improvements have been made which add to the convenience, simplicity and perhaps the accuracy of operation. Essential features of the apparatus are shown in Fig. 1. The spring, cage and work-adder are much the same as previously described. The method of recording is different. Attached to the hub of the work-adder is a lever which can swing freely. This is pulled around by the work-adder wheel until overbalanced. It then falls to trip a mercury switch, completing a circuit momentarily. The switch is connected to a relay which activates a 110 v automatic counter. It is impossible for the falling lever to register more than once for each revolution of the work-adder. The possibility of the contact dipping in the mercury pool 2 or more times per revolution because of slight play in the wheel is thus eliminated. The automatic counter obviates the necessity for tedious or even inaccurate counting of signal marks on a record.

The long spring which suspends the cage is constructed of steel music wire of 0.029 inch diameter. There are 180 turns in each spring which has an inside diameter of 13/32 inch. Galvanized screen of $\frac{1}{4}$-inch mesh is used in constructing the cages. The latter weigh about 400 g. For greatest sensitivity, the weight of the cages should be small in relation to animal weight. It has been found convenient to mount 5 complete units of the apparatus on a framework of $\frac{1}{2}$-inch aluminum rods.

In practice, adult rats weighing around 250 g are used. They produce optimal movement for the weight of our cages and the gauge of wire in the springs. There is much variation in the degree of activity of different animals. A selection is made of those which show good activity responses, the others being discarded. The selected rats are used repeatedly with appropriate rest intervals.

For studies of stimulating drugs, constant
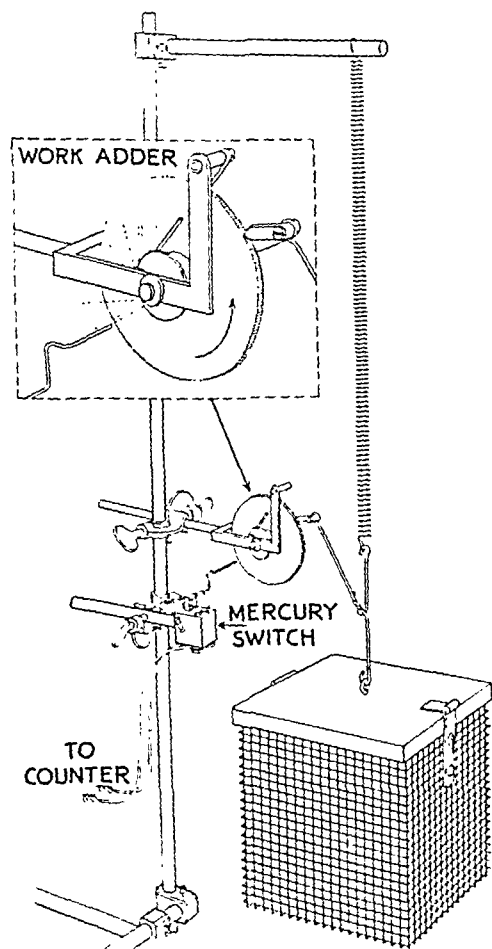
[1] Schulte, J. W., Tainter, M. L., and Dille, J. M., PROC. SOC. EXP. BIOL. AND MED., 1939, 42, 242.

[2] Schulte, J. W., Reif, E. C., Bacher, J. A., Jr., Lawrence, W. S., and Tainter, M. L., J. Pharm. and Exp. Therap., 1941, 71, 62.
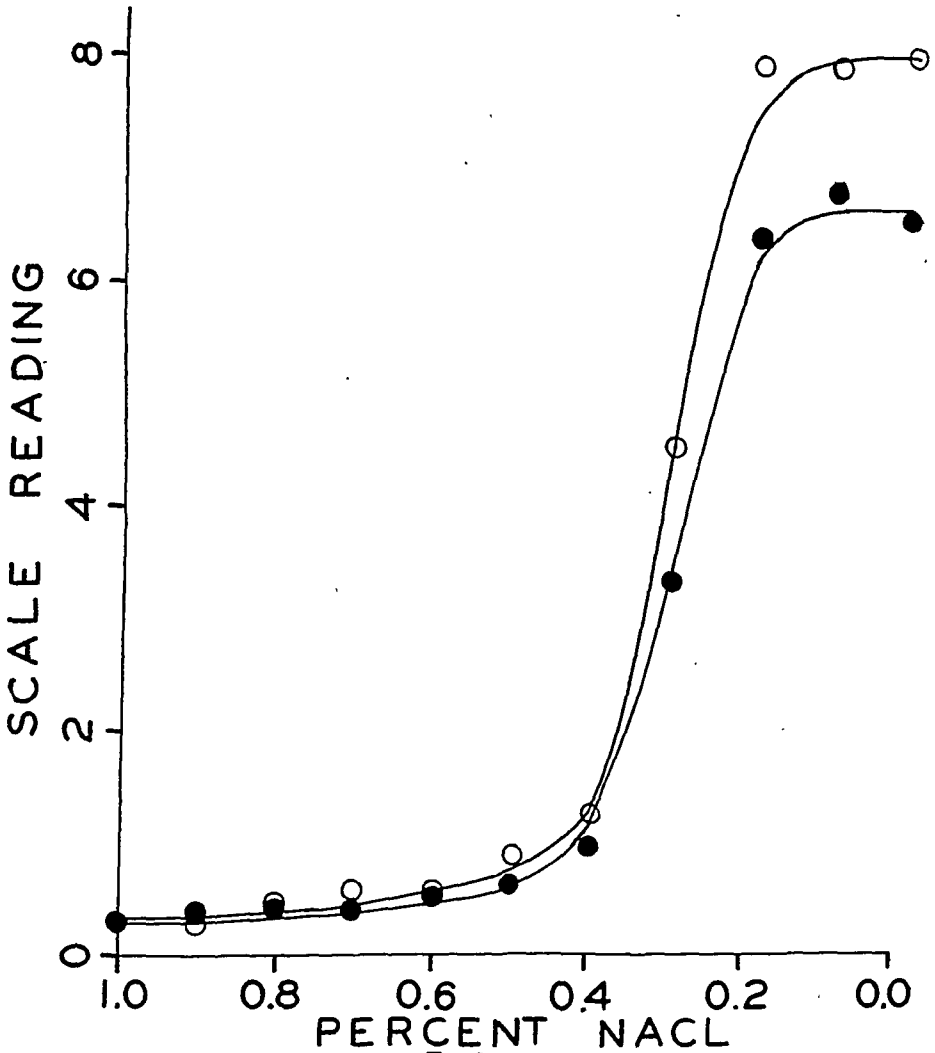
FIG. 3.

Fragility of chicken erythrocytes.   O—control;   ●—cells exposed to the test organism for 7 hr.

erythrocytes are maintained for several days under conditions favoring sepsis, the possible effect of bacterial action on the cell membranes must be considered.

Investigations of this nature are being continued to determine the generality of change in the permeable properties of cells under the influence of bacterial toxins.

Conclusions. 1. The permeability to glycerol of chicken erythrocytes exposed to a suspension of *Streptococcus β-hemolyticus* was increased after several hours of exposure to the test organism.

2. Experimental cells were no more fragile than controls.

3. The possible theoretical implications of this work are indicated.

(Continued from page iv)

light and temperature of the room are required, as suggested by Schulte *et al.* Depressant drugs may be studied in the same apparatus, but the room must be darkened to increase the normal activity. Changes, however, in studies of the latter type are much less striking.

Summary. An apparatus is described for recording the activity of rats in spring-suspended cages. The movements are automatically registered on a magnetic counter.

SECTION MEETINGS

CLEVELAND
　Western Reserve University　　　　　　　　　October 10, 1947

ROCKY MOUNTAIN
　Colorado A. and M. College　　　　　　　　　October 18, 1947

16067 P

Reproduction in the Rat on Purified Diets Containing Succinylsulfathiazole.*

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Nelson and Evans[1] have shown that placing normal adult female rats on purified diets deficient in pantothenic acid 2-3 weeks before mating or even as late as the day of mating resulted in impaired reproduction, *i.e.* failure of implantation, resorptions, or defective litters. The normality of the paired-fed controls receiving supplements of pantothenic acid eliminated the factors of inanition and of other possible specific dietary deficiencies as causes for these marked upsets in reproduc-

tion. One of the possible dietary deficiencies thus eliminated under the experimental conditions was that of pteroylglutamic acid, which has since been shown to be necessary for optimal lactation in the rat maintained on the above-mentioned control diet.[2] In the present communication we wish to report the reproductive behavior of adult females placed on a succinylsulfathiazole - containing diet (SST-diet) deficient in pteroylglutamic acid (PGA) for various periods prior to mating.

Experimental. Normal female rats (Long-Evans strain), 3 to 4 months of age were bred with normal males and placed on the deficient or control diet the day of breeding. Other groups of rats were maintained on the deficient diet for one, two, or three months and then bred as rapidly as possible. Vaginal smears were examined daily during gestation for the presence of erythrocytes, the sign that implantation has occurred; all rats were weighed at regular intervals.

1 Nelson, M. M., and Evans, H. M., *J. Nutrition*, 1946, 31, 497.

2 Nelson, M. M., and Evans, H. M., *Arch. Biochem.*, 13, 265.

acid after one to three months of the deficiency and on the relation of calorie restriction to these reproductive upsets on the SST-diet are in progress.

Summary. Adult rats maintained on a purified diet containing succinylsulfathiazole and supplemented with all the known vitamins except pteroylglutamic acid from one to 3 months before breeding showed impaired reproduction.

16068

Effect of Estrogens on Malic Dehydrogenase of Rat Liver.[*]

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The natural and synthetic estrogens are important in controlling the function of reproductive tissues, and they are also known to affect the metabolism and function of other tissues not directly concerned with reproduction. It is probable that these changes depend basically on the part played by the estrogens in certain of the enzymatic processes concerned with cellular function. As a means of obtaining information on these basic cellular processes McShan and Meyer[1] studied the effect of the estrogens on the succinoxidase system of liver and pituitary tissues. It was found that both natural and synthetic estrogens were effective inhibitors of this system, and that the inhibition was mediated through the action of the estrogens with the cytochrome oxidase of the system. This work has been extended to the study of the effect of the estrogens on the malic dehydrogenase system using the method reported by Potter[2] for the determination of this dehydrogenase. In addition results are given on the activity of malic dehydrogenase when the cytochrome c of the system is replaced by brilliant cresyl blue.

Experimental. Male and female rats of the Sprague-Dawley strain which were three to four months old were used in these experiments.

The rats were killed by decapitation and the tissues were removed immediately, weighed and placed in sharp-pointed homogenizing tubes containing 0.1 ml of glass-distilled water. These homogenizing tubes containing the tissues were kept in an ice bath during homogenization and until the tissues were placed in the Warburg flasks.

The method of Potter[2] for the determination of malic dehydrogenase was followed in these experiments. Two and one-half per cent water homogenates of the liver tissue were used throughout. Conventional Warburg flasks without side arms were employed. The main compartment of the flasks contained, in addition to the desired amount of liver homogenate, 0.3 ml of 0.5 M l-malic acid, 0.6 ml of 0.5 M glutamic acid, 0.6 ml of 0.1 M nicotinamide, 0.3 ml of a 0.5% (5 mg per ml) solution of coenzyme I (diphosphopyridine nucleotide), 0.3 ml of 4 x 10^{-4} M cytochrome c, 0.4 ml of 0.2 M phosphate buffer pH 7.4, and enough water to make a final reaction volume of 3.0 ml. The center wells of the flasks contained 0.1 ml of 2N NaOH. All solutions were made with glass-distilled water.

The cytochrome c was made from beef heart muscle according to the method of Keilin and Hartree[3] except that the final

1 McShan, W. H. and Meyer, R. K., *Arch. Biochem.*, 1946, 9, 165.

2 Potter, V. R., *J. Biol. Chem.*, 1946, 165, 311.

3 Keilin, D., and Hartree, E. F., *Proc. Roy. Soc.* (London), 1937, 122B, 298.

TABLE I.
Reproduction of Rats Maintained on Purified SST-Diets.

Time exp. diets fed	No. of rats bred	Failed implantation	Resorptions of implantations	Litters of implantations	Total No. young	Young born dead	Avg size of litter	Avg wt young
		%	%	%		%		g
		PGA—Supplemented.						
Day of Mating	10	0	0	100	87	0	8.7 (3-11)	5.9
		PGA—Deficient.						
,, ,,	12	0	0	100	112	0	9.3 (6-12)	5.5
35 Days before Mating	10	0	10	90	64	9	7.1 (2-10)	4.7
64 ,, ,, ,,	9	0	44	56	29	3	5.8 (3-9)	5.8
92 ,, ,, ,,	10	10	33	67	32	6	5.3 (2-9)	5.5

The PGA-deficient diet was composed of 24% alcohol-extracted casein, 63% sucrose, 8% hydrogenated cottonseed oil (Crisco), 4% salts,[3] and 1% succinylsulfathiazole. Crystalline vitamins per kilogram diet were: 300 µg d-biotin, 5 mg 2-methyl-1,4-naphthoquinone, 5 mg thiamine HCl, 5 mg pyridoxine HCl, 10 mg riboflavin, 10 mg p-aminobenzoic acid, 20 mg nicotinic acid, 50 mg calcium pantothenate 400 mg inositol and 1.0 g choline chloride. Control rats received the identical diet supplemented with 5.5 mg synthetic pteroylglutamic acid per kilogram diet. Each rat received weekly a fat-soluble vitamin mixture containing 800 U.S.P. units vitamin A, 115 Chick Units vitamin D, 6 mg synthetic alpha-tocopherol, and 650 mg corn oil (Mazola).

Results. Table I shows that the omission of pteroylglutamic acid from the SST-purified diet on the day of breeding did not interfere with reproduction. When the deficient diet was started one month before breeding, reproduction was slightly impaired as shown by the occurrence of resorption and of young born dead together with the slight decrease in the number of young per litter and their weight at birth. Extending the deficiency to two months before breeding resulted in marked upsets in reproductive behavior. Almost half of the animals resorbed instead of littering and the average size of the litter was markedly decreased. A further extension of the deficiency period to 3 months before breeding did not result in any greater impairment of reproduction. Additional groups of animals placed on the deficient diet at an earlier age have shown essentially the same picture.

It may be noted that no interference with implantation occurred in these PGA-deficient animals as was the case with pantothenic acid deficient animals during reproduction. A considerably longer deficiency period (1-3 months) was required to produce reproductive upsets with the SST-diet than was necessary for the pantothenic acid deficient diets (0-3 weeks). The resistance of our rats to a "folic acid" deficiency instituted after weaning has been previously pointed out.[2] Studies on the curative effects of pteroylglutamic

3 Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1941, **138**, 459.

TABLE II.

Malic Dehydrogenase Activity of Liver with Estrogenic and Androgenic Inhibitors *in Vitro*.

Q_{O_2}			
Control	Inhibitor Final molarity 10⁻⁴ M	Inhibition, %	Avg, %
	Diethylstilbestrol.		
55.9	22.5	60	44
79.7	46.9	42	
82.6	57.3	30	
	Hexestrol.		
71.8	21.3	70	75
75.3	26.6	81	
	Dienestrol.		
90.7	60.3	34	
90.8	34.2	63	
64.0	42.7	33	
65.8	38.4	32	36
79.2	47.4	40	
80.6	74.8	7	
80.6	48.2	40	
	Disodium 3.4-diphenylhexane-p,p'-dioxyacetate.		
64.0	61.4	4	
65.8	59.7	9	6
76.5	77.5	0	
85.4	76.2	11	
	No. 020.		
84.2	65.2	23	22
78.3	62.6	20	
	No. 103.		
84.2	4.8	94	93
78.3	6.1	92	
	No. 221B2.		
90.7	10.4	89	90
90.8	8.0	91	
	Na Androsterone SO₄.		
79.2	75.0	6	2
80.6	80.9	0	
80.6	80.9	0	

dehydrogenase. From the data of Table III it can be seen that there is a range of optimum dye concentration, above and below which the oxygen uptake of 0.25 ml of liver homogenate begins to decrease. This optimum range is from 0.125 to 0.75 mg of brilliant cresyl blue per flask contents contained in a total volume of 3 ml. As a result 0.5 mg of the dye was used per flask in the following experiments.

Using an amount of brilliant cresyl blue which gives maximum activity (0.1 ml of a 0.5% solution) varying amounts of tissue (from 0.05 ml to 0.35 ml) were used in an effort to determine whether the oxygen uptake was proportional to the concentration of tissue. As can be seen from the data given in Table IV, the Q_{O_2} decreases fairly rapidly with increasing amounts of liver homogenate which shows that the oxygen uptake was not directly proportional to the amount of tissue reacting. Furthermore, when each of the reagents was increased in turn to determine whether there was an insufficient amount of one of the constituents, a direct proportionality between oxygen uptake and the amount of tissue used was not obtained.

The inhibiting power of diethylstilbestrol on the malic dehydrogenase activity of liver was tested using cytochrome c alone, brilliant cresyl blue alone and a combination of this dye and cytochrome c. A final concentration of 1 x 10⁻⁴ M diethylstilbestrol was used in these *in vitro* studies. The data of Table V indicate that brilliant cresyl blue can adequately take the place of cytochrome c in the malic dehydrogenase system in that under the conditions used the oxygen uptake is as high in the presence of the dye as in the presence of cytochrome c. This is in contrast

TABLE I.
Malic Dehydrogenase Activity of Liver Homogenate.

	ml of 2.5% homogenate			
0.15	0.2	0.25	0.3	0.4
		Q_{O_2}		
	59.0	—	59.0	57.4
	81.6	—	78.8	
65.4	72.1	67.6	—	
	59.7	60.0	57.0	
	53.7	49.8	45.7	
64.4	60.9	—	—	
	106.6	101.3	—	
	90.6	93.0	—	

product was dialyzed against glass-distilled water instead of 1% sodium chloride solution. The coenzyme I was prepared in our laboratory according to the LePage modification of the Williamson and Green[4] method of preparation. Both Eimer and Amend, and Eastman 1-malic acids were used, and values obtained with each were found to be in close agreement.

Approximately 20 minutes elapsed between the time the animals were killed and the placing of the flasks in the Warburg bath at 38°C. They were allowed to equilibrate for 6 minutes, the manometer stopcocks were closed and readings were taken at 10, 20 and 30 minutes. Q_{O_2} values were based on the average of these 3 readings.

Results and Discussion. A series of determinations was made to ascertain whether the oxygen uptake was proportional to the amount of liver homogenate used. In these runs, quantities of liver varying from 0.15 to 0.4 ml of 2.5% homogenate were used. The data presented in Table I show that the oxygen uptake of the malic dehydrogenase system is essentially proportional to the amount of liver tissue used within a narrow range of wet weight of tissue. There were several determinations made, however, in which an exact proportionality was not obtained.

Several studies were made employing various estrogenic compounds and sodium androsterone sulfate[†] as *in vitro* inhibitors of malic dehydrogenase activity of rat liver.

The test system contained 0.25 ml of a 2.5% liver homogenate and 0.15 ml of 2 x 10⁻³ M solution of the estrogenic compound. The estrogens employed were diethylstilbestrol, hexestrol, dienestrol and disodium 3,4-diphenylhexane-p,p'-dioxyacetate and benzestrol estrogenic compounds No. 020 (HO-C_6H_4-CH_2-CHC_2H_5-CH_2-C_6H_4-OH), No. 103 (HO-C_6H_4-$CHCH_3$-CH_2-CHC_3H_7-C_6H_4-OH) and No. 221B-2 (HO-C_6H_4-CHC_2H_5-CHC_2H_5-$CHCH_3$-C_6H_4-OH). These inhibitors, with the exception of disodium 3,4-diphenylhexane-p,p'-dioxyacetate, which is water soluble, were dissolved in 0.5 ml water that contained 0.03 ml of 2N NaOH after which sufficient glass-distilled water was added to make 5 ml. Control flasks were run that contained 0.15 ml of a solution containing 0.03 ml of 2N NaOH per 5 ml, and the addition of this weakly basic solution had no effect on the malic dehydrogenase values.

The data presented in Table II indicate that the estrogenic compounds are efficient inhibitors of the malic dehydrogenase activity of rat liver *in vitro*. The 8 compounds used are listed in the order of increasing inhibitory power: Androsterone sulfate 2% inhibition, disodium-3,4-diphenylhexane - p,p'- dioxyacetate, 6%, No. 020 (benzestrol) 22%, dienestrol 36%, diethylstilbestrol 44%, hexestrol 75%, No. 221B2 (benzestrol) 90%, and No. 103 (benzestrol) 93%.

To determine whether malic dehydrogenase itself was inhibited, or whether the inhibition was effected through inhibition of cytochrome oxidase, cytochrome c was replaced by brilliant cresyl blue. Preliminary results indicated that the cytochrome c of the malic dehydrogenase system can be completely substituted for by brilliant cresyl blue. On the basis of these data and before doing further work on the inhibition of this system by the estrogens, it was decided to determine the amount of the dye required for optimum activity of malic

4 Williamson, S., and Green, D. E., *J. Biol. Chem.*, 1940, **135**, 345.

TABLE V.

Inhibition by Diethylstilbestrol of Malic Dehydrogenase of Liver Using Cytochrome c and Brilliant Cresyl Blue.

	Q_{O_2}					
	Cytochrome c		Cresyl blue		Cytochrome c-Cresyl blue	
	Control	Inhibitor*	Control	Inhibitor*	Control	Inhibitor*
	84.3	34.4	82.2	62.9	—	—
	62.9	3.5	69.0	61.6	—	—
	70.0	13.0	73.9	71.0	77.5	71.3
	75.3	27.2	78.7	75.6	71.3	68.9
	71.2	5.9	67.9	59.8	64.0	61.9
	71.2	7.1	69.2	58.3	64.0	60.4
	74.8	11.8	75.1	63.0	70.1	67.8
Avg	72.8	14.7	73.7	64.6	69.4	66.1

* Diethylstilbestrol, final concentration 1 x 10-4 M.

action. This does not seem unreasonable as cytochrome oxidase, through which the inhibition was shown to be effected, is known to catalyze the oxidation of certain phenolic compounds. It is suggested that there is an affinity, therefore, between the active centers of the enzyme and phenolic groups. Thus, it appears that the phenolic groups of the estrogenic compounds combine with the active centers and remain attached to the enzyme which prevents the latter from acting. This mechanism was shown previously to account for the inhibition of the succinoxidase system by estrogens.[1]

Summary. The malic dehydrogenase activity of liver homogenate was shown to be proportional to the amount of tissue used.

It was found that the cytochrome c of the malic dehydrogenase system could be replaced by brilliant cresyl blue. The maximum activity of the dehydrogenase was obtained when the concentration of the dye ranged from 0.125 mg to 0.75 mg per flask.

The inhibitory action of one androgenic and 7 estrogenic compounds was tested on liver malic dehydrogenase *in vitro.* Stilbestrol and benzestrol estrogens are effective inhibitors, but little inhibition occurs when the phenolic groups of the stilbestrol type compounds are replaced by oxyacetate groups as for example in disodium 3,4-diphenylhexane-p,p'-dioxyacetate. In addition sodium androsterone sulphate which contains an alcoholic in place of a phenolic group shows very little inhibitory action. It appears that the presence of phenolic groups are necessary for inhibition to take place.

It was concluded also from inhibitor studies with diethylstilbestrol, in which brilliant cresyl blue was substituted for cytochrome c, that diethylstilbestrol exerts its inhibitory action on cytochrome oxidase rather than malic dehydrogenase.

TABLE III.
Malic Dehydrogenase Activity of Liver with Various Concentrations of Brilliant Cresyl Blue.

Brilliant cresyl blue, mg/flask	Q_{O_2}					
	Run I	II	III	IV	V	Avg
—	3.9	0.0	4.7	6.2		4
.0125	25.6	—	—	—		26
.025	52.8	—	—	—		53
.0375	62.1	—	—	—		62
.05	64.4	—	—	—		64
.0625	76.0	61.3	66.7	—		68
.125	76.0	77.6	72.9	—		76
.25	72.9	77.6	71.4	77.6		75
.325	71.4	69.1	77.6	—		73
.5	77.6	77.6	76.0	77.6	74.5	77
.75	76.0	77.6	69.8	71.4	—	74
1.0	68.3	55.1	77.6	62.9	55.9	64
2.0	57.4	62.9	60.5	59.8		60
3.0	66.0	59.8	65.2	53.5		61
4.0	49.7	77.6	62.9	43.5		58

TABLE IV.
Malic Dehydrogenase Activity with Brilliant Cresyl Blue* and Varying Concentrations of Liver Homogenate.

	Q_{O_2}					
0.05 ml	0.1 ml	0.15 ml	0.2 ml	0.25 ml	0.3 ml	0.35 ml†
91.7	92.4	85.7	77.0	77.0	74.1	
—	74.2	74.6	—	71.5	66.4	57.5
106.7	90.6	108.1	82.0	66.4	—	
—	—	85.6	80.9	71.2	—	
—	88.9	74.2	85.0	71.0	—	
71.2	87.6	73.9	71.6	60.7	—	
71.2	68.2	68.4	58.5	57.3	60.9	
106.9	74.1	62.4	67.8	68.0	56.1	
Avg 89.5	82.3	79.1	74.7	67.9	64.4	57.5

* Concentration of brilliant cresyl blue was 0.5 mg per flask.
† Liver homogenate was 2.5%.

to the succinoxidase system in which the activity is decreased considerably when the dye is substituted for cytochrome c.[5,1] The fact that this dye is autoxidizable in the presence of oxygen permits the malic dehydrogenase system to function without the action of cytochrome oxidase. Thus, if the estrogen inhibition is taking place by way of cytochrome oxidase, the addition of brilliant cresyl blue to the system should result in the malic dehydrogenase being essentially as active in the presence as in the absence of the inhibitor. The data of Table V show that this is the case. Diethylstilbestrol in a final concentration of 1×10^{-4} M is an effective in-

hibitor of the malic dehydrogenase system when cytochrome c is used, but when it is replaced with brilliant cresyl blue there is little inhibition, and there is even less when both cytochrome c and dye are present. These results support the concept that the estrogens inhibit the malic dehydrogenase system through action with the cytochrome oxidase of the system and not by direct action with the malic dehydrogenase.

Furthermore, only those compounds that have phenolic groups inhibit the malic dehydrogenase system, and when these groups are replaced by oxyacetate groups little, if any, inhibition occurs. Also sodium androsterone sulphate which contains an alcoholic in place of a phenolic group does not show inhibitory

5 Weil-Malherbe, H., *Biochem. J.*, 1937, **31**, 299.
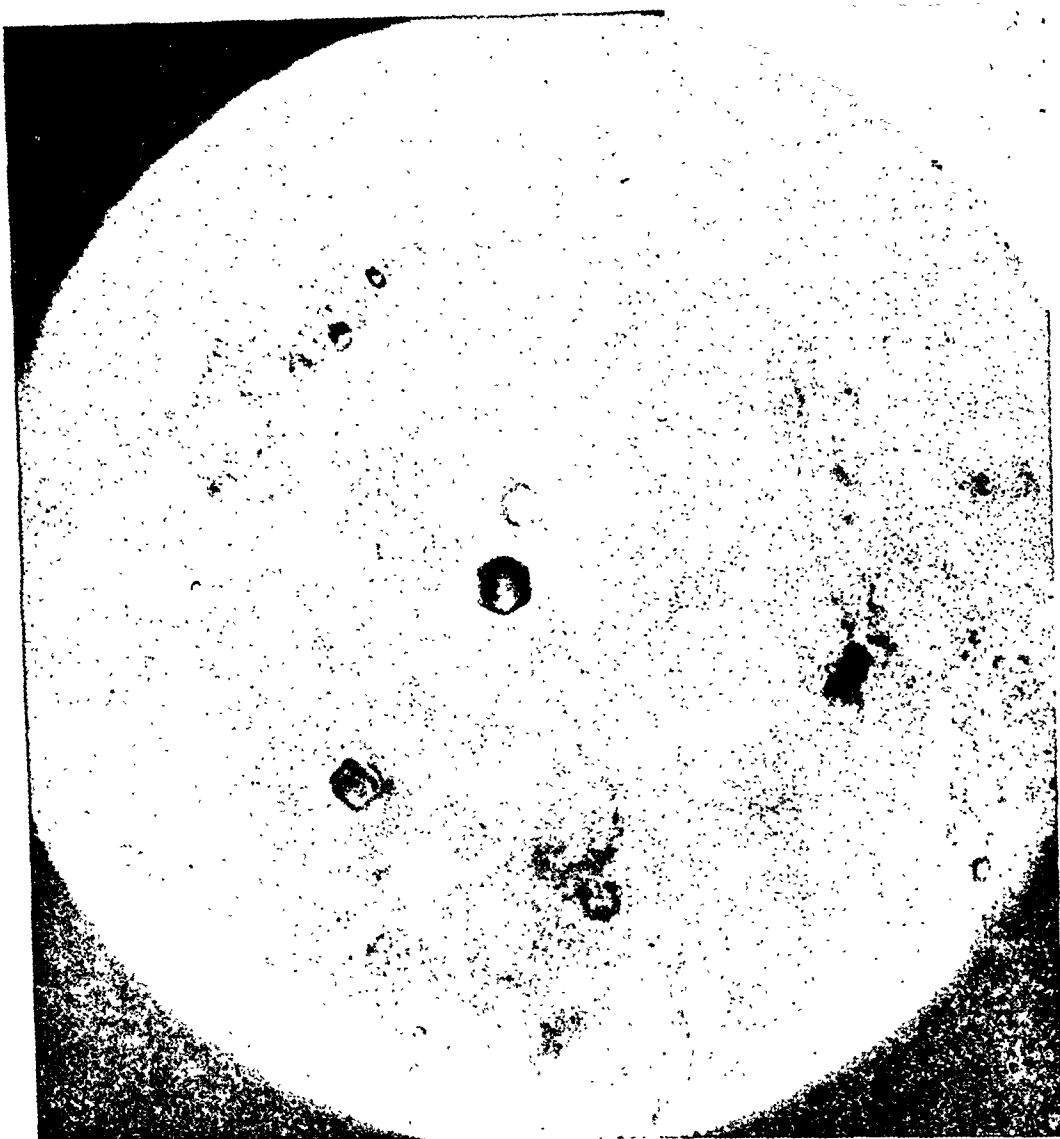
Fig. 1.

The solution contained 5.73 mg Fe/cc and 0.858 mg P/cc, or a ratio of 3.7 Fe : 1 P atom. The phosphorus appeared to belong to the iron hydroxide-phosphate micelles of ferritin since all of the phosphorus and iron could be dialyzed away on treating this ferritin with hydrosulfite at pH 4.6. This material was higher in P than was horse ferritin where the ratio is 8 or 9 Fe to 1 P atom. The ferritin contained 16.5% by dry weight of iron.

Magnetic measurements on this sample gave a value of 3.90 Bohr magnetons (B.M.) per g atom of Fe, the susceptibility being measured at 25°C. This value agrees within the limits of error with the value obtained for ferritin iron from horse and dog.[3] A sample of normal human ferritin kept in the ice-box for 3 years gave a value of 3.94 B.M.

[3] Michaelis, L., Coryell, C. D., and Granick, S., J. Biol. Chem., 1943, 148, 463.

16069

The Presence of Ferritin in the Duodenal Mucosa and Liver in Hemochromatosis.

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The etiology of hemochromatosis is unknown.[1] Recently, work on ferritin has indicated that this substance is a normal iron storage protein of the body. Ferritin, by virtue of its storage property also appears to function in the regulation of iron absorption from the gut. The opportunity was presented of examining whether a relationship might exist between factors governing ferritin formation and those which lead to huge deposits of iron in hemochromatosis.

Case history: Presbyterian Hospital unit number 861951. Admitted March 15, died May 6, 1947.

The patient was a housewife of 56, who had had diabetes mellitus and an enlarged liver for seven years. *B. coli* peritonitis, and bilateral oophorectomy in 1945. She developed congestive heart failure a year before admission, without valve lesions or hypertension. Bronzing of the skin was not noticed until it was called to her attention. During her stay in the hospital auricular fibrillation and ascites appeared. Her diet was difficult to control, as she was disoriented at times. Small doses of standard insulin were used. The laboratory findings were: RBC 4,400,000, Hgb. 13.0 g, WBC 11,900, differential normal. Serum alkaline phosphatase 10.1 Bodansky units, urea N 14 mg%, bilirubin, trace, cholesterol 213 mg%, albumin 3.2%, globulin 2.4%, cephalin flocculation +, prothrombin time 24 sec., blood sugar varied from 70 to 200 mg%, serum CO_2 content 55 vol.%. A biopsy from the left upper quadrant of the abdomen showed normal muscle, but hemosiderosis of the skin compatible with hemochromatosis. She died after 6 weeks, in congestive failure.

Autopsy number 15,292: Death 12:15 a.m., autopsy 2:00 a.m. Organs placed in ice-box, delivered to the Rockefeller Institute 9 a.m. The findings were typical of hemochromatosis, both in the gross and microscopically, with marked cirrhosis of the liver, and fibrosis of the myocardium. There was an old mural thrombus at the apex of the left ventricle, infarcts of liver, spleen and right kidney, and an embolus in the left common iliac artery. A terminal bronchopneumonia and other incidental findings were noted. The liver weighed 2,210 g, spleen 220 g, and heart 340 g. The pancreas showed moderate siderosis, the islets were not remarkable. The pituitary showed a large scarred area in the pars anterior.

Experimental. Ferritin was isolated from *the liver and spleen by a procedure described* previously.[2] The human ferritin precipitates from solution as brown spheroids, *instead of well-defined crystals when* $CdSO_4$ *is added to a solution of ferritin. Further purification was attempted by dissolving the* $CdSO_4$-*cipitate in ammonium sulfate (4 g/100 cc). On increasing the ammonium sulfate concentration up to 10 g/100 cc, a brownish slimy material could be centrifuged away. The remaining deep brown solution precipitated out completely on dialysis against distilled water. This precipitate was dissolved by bringing it to pH 5 with acetate buffer and the properties of the resulting solution were examined.*

Addition of $CdSO_4$ to an aliquot of the solution resulted in a precipitate of brown spheroids with no suggestion of crystalline shape, indicating that this material was still impure.

1 Sheldon, J. H., *Hemochromatosis*, Oxford University Press, London, 1935.

2 Granick, S., *J. Biol. Chem.*, 1943, **149**, 157.

16070

Sparing Action of Protein on Pantothenic Acid Requirement of Rat. III. Fibrin as the Protein Component.*

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The sparing action of protein on the pantothenic acid requirement of the rat was first demonstrated by Nelson and Evans[1] with purified diets, deficient in pantothenic acid, containing 64% instead of 24% casein. When intermediate levels (24%, 34%, 44%, 54%, and 64%) of casein were used, the sparing action as judged by growth, survival and urinary excretion of pantothenic acid of deficient rats was proportional to the casein level.[2] The presence of small amounts of "combined" pantothenic acid in the casein used (and in all types of purified casein tested) prompted a study of the effects of 24% casein diets supplemented with the amounts of pantothenic acid occurring in the casein of the 44% and 64% casein diets. The results showed that the pantothenic acid content of the purified casein used, while exercising an effect, could not fully account for the protective action of the high casein diets.[2]

However, it was felt highly desirable that a protein free from or considerably lower in pantothenic acid than casein be used in the further investigation of this problem. Preliminary assays indicated that beef blood fibrin, purified by washing with water or with dilute acid, was extremely low in pantothenic acid. The present communication reports the growth and survival of pantothenic acid deficient rats maintained on diets containing two levels of purified fibrin, 24% and 48%, and on the corresponding casein-containing diets.

Experimental Procedure. Dried crude beef blood fibrin (Armour and Company) was purified by washing with water for one week.† One kg of crude fibrin was placed in a 20-gallon crock and 15 gallons of water (either tap or distilled) added to it. The mixture was stirred thoroughly and allowed to settle. The supernatant liquid was siphoned off 2-4 times daily and fresh water added. Ten cc of chloroform were used with each change of water as a preservative. After 5-7 days of washing, the supernatant was drained off and the fibrin filtered through a large Buchner funnel. Suction was maintained for several hours or overnight if necessary to dry out the fibrin cake. It was then broken up by sieving and dried for 24 hours in a low temperature oven (50°C). After grinding, the fibrin is ready for use in the diets.

Microbiological assays for pantothenic acid were carried out by the method of Hoag, Sarett, and Cheldelin[3] with crystalline B vitamins substituted for the rice bran extract and autolyzed yeast in the assay medium. Enzymatic digestion with clarase or mylase P for 24 hours at 37°C[4] followed by the 24-hour assay procedure was used for the purified fibrin and casein. The water-washed fibrin used in the diets averaged 0.5 μg pantothenic acid per g whereas the alcohol-extracted casein contained 1.5-1.8 μg per g. There was considerable variation in the pantothenic acid content of different lots of fibrin after washing and additional purification was sometimes

[1] Nelson, M. M., and Evans, H. M., PROC. SOC. EXP. BIOL. AND MED., 1945, 60, 319.

[2] Nelson, M. M., van Nouhuys, F., and Evans, H. M., *J. Nutrition,* 1947, 34, 189.

[3] Hoag, E. H., Sarett, H. P., and Cheldelin, V. H., *J. Ind. Eng. Chem., Anal. Ed.,* 1945, 17, 60.

[4] Ives, M., and Strong, F. M., *Arch. Biochem.,* 1946, 9, 251.

Reprecipitation of the brown solution with $CdSO_4$ yielded about 1.2 cc of packed globules per 100 g of fresh liver. This is the highest content of ferritin yet obtained from human livers.[2] Ferritin was also isolated from the spleen and had properties of solubility and precipitability with $CdSO_4$ similar to those of the liver material. However, only about 0.11 cc of packed reprecipitated ferritin globules per 100 g fresh spleen were isolated.

A homogenate, prepared from a piece of liver by grinding in a Waring blender and further comminuting in a TenBroeck grinder was subjected to magnetic measurements. It had a value of 4.0 B.M. per g Fe atom. The iron in this homogenate consisted for the most part of ferritin and of hemosiderin granules, with a relatively negligible contribution from the iron porphyrin pigments. It is interesting that this value is not essentially different from the value of ferritin itself, probably indicating that the hemosiderin granules in the liver contain iron in the same structural configuration as in ferritin. A somewhat lower value for hemosiderin granules was obtained from horse spleen (B.M.$=$3.4).[3]

The duodenal mucosa was tested for ferritin by adding $CdSO_4$ to scrapings of the mucosa on a glass slide and examining it after 24 hours. Brown crystals of ferritin were observed in this material (Fig. 1). The crystals were better defined, with sharper outlines, than any human ferritin crystals seen previously. The presence of the crystals is evidence for the presence of ferritin in the duodenal mucosa. Normal human mucosa has not yet been examined for the presence of ferritin.

Discussion. The fact that ferritin can be found in the duodenal mucosa, spleen, and especially abundantly in the liver, indicates that hemochromatosis is not brought about by a failure to produce the specific protein apoferritin. The magnetic measurements on the ferritin of the liver also indicate that the structural configuration of the iron hydroxide-phosphate of the ferritin is normal, although its phosphorus content appears to be somewhat high.

The idea has often been suggested that the mechanism governing the intake of iron through the intestinal mucosa might be damaged or non-functional in hemochromatosis. If ferritin is assumed to play a role in the regulation of iron absorption[4] then the presence of ferritin in the duodenal mucosa does not support the idea that the regulatory mechanism of iron-absorption is directly affected.

In general, the normal blood picture in hemochromatosis and also the high level of iron in the serum suggest that the transport of iron is adequate.

The extremely high ferritin content of the liver and the normal magnetic values for ferritin iron indicate that the mechanism for iron storage is functioning at capacity.

Conclusion. The cause of hemochromatosis does not appear to be a defect in the formation of ferritin in the duodenal mucosa or the liver.

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4 Granick, S., *J. Biol. Chem.*, 1946, **164**, 737.

TABLE I.
Effect of Casein and Fibrin Levels on Growth and Survival of Pantothenic Acid Deficient Rats.

Dietary protein	No. of rats	Survival			Avg body wt Day 60 g	Survival		Avg body wt Day 90 g
		Day 30 %	Day 45 %	Day 60 %		Day 75 %	Day 90 %	
24% fibrin	30	83	60	23	72	3	3	73
48% "	30	97	77	57	86	43	33	111
24% casein	20	100	80	60	93	50	40	117
48% "	20	100	95	85	105	55	45	132

tent of the washed fibrin is the obvious factor responsible for the accentuation, although other possibilities such as the presence of accentuating factors, decreased intestinal synthesis, or the lack of biotin and folic acid[7,8] cannot be eliminated without investigation. Sarma, Snell and Elvehjem[9] have reported an accentuation of pyridoxine deficiency when fibrin-containing diets were used and believed that the accentuation was greater than could be accounted for by the decreased pyridoxine content of the fibrin diets.

Fibrin and casein are similar in amino acid content[10] but are markedly different in regard to phosphorus content. Fibrin is extremely low in phosphorus and is frequently used for studies of phosphorus deficiency, while casein a conjugated phosphoprotein, contains somewhat less than 1% phosphorus. Doubling the proportion of fibrin in the diet does not significantly affect the phosphorus content whereas doubling the proportion of casein markedly increases the phosphorus concentration of such diets. The phosphorus content of the diets used (calculated from the phosphorus

analyses of purified fibrin and casein[‡] and the composition of the salt mixture) are as follows: 24% fibrin diet—0.6%; 48% fibrin diet — 0.6%; 24% casein diet — 0.7%; and 48% casein diet—0.9%. The fact that an increased phosphorus content is unnecessary for the sparing action of high fibrin diets indicates that phosphorus can be eliminated as a possible factor in the sparing action of protein on the pantothenic acid requirement of the rat.

Summary. The sparing action of protein on the pantothenic acid requirement of the rat has been confirmed by using a protein considerably lower in pantothenic acid, washed beef blood fibrin, instead of casein as the protein component of the diet. Despite the accentuation of the pantothenic acid deficiency produced by substituting purified fibrin for casein at the 24% level, doubling the fibrin level resulted in significantly better growth and survival. The differences in growth and survival between the two protein levels in the fibrin-containing diets were more pronounced than those observed for the casein-containing diets. The protective effects of the high fibrin diet indicated also that phosphorus can be eliminated as a possible factor concerned in the sparing action.

[7] Wright, L. D., and Welch, A. D., *Science*, 1943, 97, 426.

[8] Wright, L. D., and Welch, A. D., *J. Nutrition*, 1944, 27, 55.

[9] Sarma, P., Snell, E. E., and Elvehjem, C. A., *J. Biol. Chem.*, 1946, 165, 55.

[10] Block, R. J., and Bolling, D., *The Amino Acid Composition of Proteins and Foods*, 1945, Chapter X, p. 300, Charles C. Thomas, Springfield, Ill.

[‡] The phosphorus analyses were carried out by Miss Genevieve Beach through the courtesy of D. H. Copp. The water-washed fibrin contained 0.045% and the alcohol-extracted casein 0.61% phosphorus.

necessary. Other methods of purification are being investigated in order to prepare fibrin completely free of pantothenic acid for balance studies.

The basal diet contained 24% alcohol-extracted casein or water-washed fibrin, 64% sucrose, 8% hydrogenated cottonseed oil (Crisco) and 4% salts No. 4.[5] The proportion of casein or of fibrin in the higher protein diets was increased to 48% at the expense of the sucrose. All diets contained the following crystalline vitamins per kg diet: 5 mg 2-methyl-1,4-naphthoquinone, 5 mg thiamine HCl, 5 mg pyridoxine HCl, 10 mg riboflavin, 10 mg p-aminobenzoic acid, 20 mg nicotinic acid, 400 mg inositol, and one g choline chloride. Each rat received weekly a fat-soluble vitamin mixture containing 650 mg corn oil (Mazola), 800 U.S.P. units vitamin A, 115 Chick Units vitamin D, and 6 mg alpha-tocopherol. The basal diet containing 24% casein is the high-carbohydrate diet used in previous studies[1,2] with the addition of 2-methyl-1,4-naphthoquinone to the vitamin mixture. The previous weekly allowance of the fat-soluble vitamins has been doubled.

In the first series of animals, 20 litters of male rats (Long-Evans strain) were divided into 4 equivalent groups and placed on the pantothenic acid deficient diets containing 24% or 48% casein or fibrin at weaning. An additional series of 10 litters of male rats were divided into equivalent groups and placed on the 2 fibrin diets at weaning. All animals were maintained in individual cages provided with screens to prevent coprophagy.

Results and Discussion. The growth of littermates (20 rats per group) maintained on 24% and 48% casein or fibrin diets deficient in pantothenic acid is shown in Fig. 1, while Table I presents the data on growth and survival for the combined series of animals. The sparing effect on growth and survival of doubling the casein level (24% to 48%) is evident although the effect is not as great as it would be in a longer experimental period or with higher casein levels.[2] The substitution of purified fibrin in the 24% casein diet resulted in
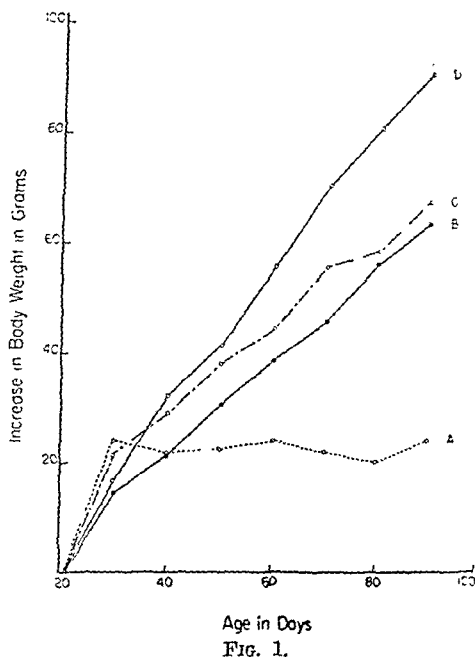
FIG. 1.

Growth of pantothenic acid deficient rats maintained on purified diets varying in casein or fibrin level.
A. 24% fibrin diet.
B. 48% ,,   ,,
C. 24% casein ,,
D. 48% ,,   ,,

a marked accentuation of the deficiency, shown in the greatly decreased growth and survival in comparison to all other diets. Despite the severity of the deficiency, doubling the fibrin level (48% fibrin diet) resulted in significantly increased growth and survival. In fact, the differences in growth and survival were greater than those between the 24% and 48% casein diets during the short experimental period.

The accentuation of the deficiency observed on the 24% fibrin diet was not due to the lack of any essential factors in the purified fibrin. Control rats receiving the 24% fibrin diet supplemented with calcium pantothenate grew slightly faster than littermates receiving the supplemented 24% casein diet. This is in agreement with the findings of Risser[6] that fibrin, both crude and pharmaceutical grades, was somewhat higher in biological value than casein. The decreased pantothenic acid con-

5 Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1941, **138**, 460.

6 Risser, W. C., *J. Nutrition*, 1946, **32**, 485.

maintained;[3,4] (2) androgens have a reparative influence;[5] (3) the results of one especially favorable case of testis graft in a female showed localized inhibition of the oviduct, more extensive persistence of the wolffian duct.

Some new observations contribute complementary data on the manner of action of the testicular secretion. In 3 males unilaterally castrated between the ages of 19 days 6 hours and 20 days 21 hours, the prostate and the external genitalia are well developed, but the single testis acts less effectively on the primordia of the opposite side. In a fetus with only a right testis, of somewhat under normal size, the vas deferens is present only on the right side, while on the left, a uterine horn persists. It follows that after early unilateral gonadectomy the action of the fetal testis is relatively localized. A single testis or a fragment of a testis (as appears in the case of an incomplete castration) does not seem to undergo compensatory secretory hypertrophy;

with regard to this point, it should be remembered that the hypophysis is not indispensable for embryonic sexual differentiation.[9,10,11] Lastly, some of our observations suggest that more testicular secretion is required to completely inhibit the mullerian ducts, than to maintain the wolffian ducts.

Conclusion. In the male rabbit, early fetal castration (before the 21st day) prevents the formation of male secondary sex characters such as the prostate. At a later stage (24 days) established primordia continue their development even after ablation of the testes; the resulting structures are usually subnormal. The effects induced by testicular secretions are sometimes regionally restricted. Earlier observations on the differentiation and persistence of female genital ducts after early orchidectomy are confirmed and extended.

5 Jost, A., *C. R. Soc. Biol.*, 1947, 141, 275.

9 Ancel, P., *Les Hormones Sexuelles*, Colloque Singer Polignac, Paris, 1937, Hermann.

10 Wolff, E., and Stoll, R., *C. R. Soc. Biol.*, 1937, 126, 1215.

11 Fugo, N., *J. Exp. Zool.*, 1940, 85, 271.

16072

The Lymphoid Tissue and Antibody Formation.

JAMES B. MURPHY AND ERNEST STURM.

From the Laboratories of the Rockefeller Institute for Medical Research, New York City.

Several early investigators in immunology considered the possibility that the spleen was the main site of antibody formation, but conclusive evidence for this was not found. Hektoen,[1] using the well-known fact that X-ray produces a destructive effect on all lymphoid tissue, demonstrated that antibody formation is restrained to a marked degree in X-rayed animals. He concluded from this observation that the spleen, lymphoid tissue and bone marrow were the principal sites of immune bodies. Murphy and Sturm[2] confirmed Hek-

toen's findings but considered that they had eliminated the bone marrow as of first importance in the process. This conclusion was based on the fact that depletion of the lymphoid tissue was effected by repeated, small doses of X-ray of low penetration which produced no detectable damage to the bone marrow or other tissues. Yet animals treated in this manner were found deficient in the production of antibodies. In addition, these investigators demonstrated that rabbits, after stimulation of the lymphoid system by dry heat treatment, had an enhanced ability to produce immune substances. Hussey[3] in this

1 Hektoen, L., *J. Infect. Dis.*, 1915, 17, 415; 1918, 22, 28; 1920, 27, 23.

2 Murphy, J. B., and Sturm, E., *J. Exp. Med.*, 1925, 41, 245.

3 Hussey, R. G., PROC. SOC. EXP. BIOL. AND MED., 1921, 19, 22.

16071

The Age Factor in the Castration of Male Rabbit Fetuses.

A. Jost. (Introduced by E. Witschi.)

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Muséum National d'Histoire Naturelle, Paris.

In previously reported experiments on intra-uterine surgical castration of rabbits, it was ascertained that the fetal gonads play some part in somatic sexual differentiation. In controls, the first somatic sexual differences appear on the 20th day;[1] and prostate buds appear at 21 days. At 27-28 days, genital organogenesis is practically completed; all control and experimental fetuses were dissected and preserved at that age. The following observations were published recently: (1) males gonadectomized at 23 days (4 cases) acquired nearly all male characters, but the deferent ducts and ampullae were partly involuted;[2,3] (2) in males castrated at 22 days (2 cases) the wolffian ducts had disappeared, but vestiges of mullerian ducts were present; the prostate was reduced and the external genitalia were abnormal.[4] (3) In a group of males castrated at 21 days, 3 out of 8 retained some wolffian vestiges; the other 5 had none; the mullerian ducts persisted and had differentiated into mullerian vagina, uterine horns, and sometimes, oviducts. In microscopic sections, the external genitalia presented a feminine aspect. Only 2 rudimentary prostatic buds had developed; they correspond to the cranial prostate, which is first to appear in normal development. (4) There were great similarities between fetuses of both sexes castrated at this age, but only the males had prostatic buds.[5]

It was the aim of the now to be presented new series of experiments to determine: (1) whether male fetuses may acquire and retain normal male characters, if castrated after the

23rd day, and (2) whether their prostate development may be suppressed completely, if castrated before the 21st. The following results were obtained.

Two males, gonadectomized at 24 days, continued to develop according to the male type, retaining their deferent ducts and ampullae. The size of the prostate is somewhat below normal.

In fetuses operated before the age of 21 days, the following conditions were observed: 3 males castrated at 20 days developed and retained female genital tracts, but also had 2 prostatic buds. In 2 males gonadectomized at 18 days 19 hours, and 19 days 5 hours, *i.e.*, before the initiation of somatic sexual differentiation, prostatic development was entirely suppressed. Another male, castrated at the same age, and treated with testosterone propionate, exhibited a large prostate.

Thus, in order to suppress completely the formation of prostates, it is necessary to castrate the embryos before the development of their primordia. After primordia have reached a certain stage of development, especially from the 23rd day on, they continue growing and branching, in spite of the absence of the testes. However, they are somewhat reduced in size; this point should be checked by a quantitative study like that devoted to the rat by Wells.[6] One may wonder whether Moore's opossums were already too old for prostate development to be suppressed when they were gonadectomized.[7]

Three groups of facts suggest that the testis exerts its influence by means of actual secretions: (1) in males unilaterally castrated at 22 or 23 days, the wolffian ducts are fully

1 Jost, A., *C. R. Soc. Biol.*, 1943, **137**, 586.
2 Jost, A., *C. R. Soc. Biol.*, 1946, **140**, 774.
3 Jost, A., *C. R. Soc. Biol.*, 1946, **140**, 938.
4 Jost, A., *C. R. Soc. Biol.*, 1947, **141**, 126
5 Jost, A., *C. R. Soc. Biol.*, 1947, **141**, 135.

6 Wells, L. J., *Proc. Soc. Exp. Biol. and Med.*, 1946, **63**, 417.
7 Moore, C. R., *J. Exp. Zool.*, 1943, **94**, 415.

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16072

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laboratory found that if the lymphoid system of guinea pigs is kept in a depleted state by X-ray following a sensitizing injection of horse serum, the animals show no anaphylactic manifestations on receiving a second injection of serum. In such animals the antigen continues free in the circulation as long as the X-ray is continued. McMaster and Hudack[4] have clearly demonstrated that antibodies may be formed in lymph nodes but their experiments did not eliminate the possibility that cells other than lymphocytes participated in the reaction. Ehrich and Harris[5] have extended these observations and on the basis that the tissue response accompanying the formation of antibodies in the nodes is predominately a lymphoid one, they consider these cells as the important factor in the development of immune substances. Harris, Grimm, Martens and Ehrich,[6] in a study of the antibodies in the lymph, found when the lymphocytes are separated from the lymph plasma that the titer of the cell extract was substantially and consistently higher than that of the lymph plasma. This is taken to indicate that the lymphocytes either produce antibodies or take them up from lymph plasma. As no evidence for the latter was found, they believe that the lymphoid cells are the responsible agents. Dougherty, Chase and White[7] noted that the antibody content of the blood is increased following the administration of adrenal cortical extract, a fact which they attributed to the destructive action of the hormones on the lymphocyte with the release of the antibodies supposedly formed in the cytoplasm of these cells. Philips, Hopkins and Freeman[8] found no such increase when the lymphoid tissue is destroyed by nitrogen mustard and such treatment may result in an actual depression of the production of antibodies.

In the present investigation an estimate has been made of the antibody formation in animals with extreme hypertrophy of the lymphoid system which follows the removal of the adrenal glands. The release of the antibodies from the lymphocyte under these conditions would be due to the normal shedding of the cytoplasm as the cell matures. In the absence of the adrenals there can be no question of the cortical hormones playing any part in the reaction.

Circulating Lymphocytes in Adrenalectomized Rabbits. The circulating lymphocytes show a definite but not marked rise following removal of the adrenals. This increase is in the average range of 4000 cells per mm³, which is considerably less than that observed for mice and rats. However, if adrenalectomized rabbits are given an antigen, the lymphocyte count shows a marked increase up to an average of over 14,000 cells while normal rabbits under the same condition have only a slight increase.

Antibody Formation in Adrenalectomized Rabbits. Experiment 1. The adrenals were removed from 2 rabbits and immediately afterwards these animals and normal controls were given 10 cc of horse serum intraperitoneally. Ten days later, all were bled and the sera tested for precipitins. The sera from the adrenalectomized rabbits gave massive precipitates in the low dilutions, and definite positive reactions in dilutions of 1 to 640 in one serum and in dilutions of 1 to 2560 in the other. The serum from the control gave far less precipitate in low dilutions and there were no precipitates in dilutions higher than 1 to 320.

Exp. 2. The adrenals were removed from 7 rabbits and between 2 and 3 weeks later these with four controls were given five intravenous injections of horse serum, consisting of 1 cc, at 2-day intervals. All of the rabbits were bled 10 days following the last injection and the sera tested for precipitins. The results are given in Table I. It will be noted that all of the sera from adrenalectomized rabbits gave definite precipitates in dilutions up to 1:2560 and 4 were positive in 1:5120. Only

4 McMaster, P. D., and Hudack, S. S., *J. Exp. Med.,* 1935, **61**, 783.

5 Ehrich, W. E., and Harris, T. N., *J. Exp. Med.,* 1942, **76**, 335; *Science,* 1945, **101**, 28.

6 Harris, T. N., Grimm, E., Mertens, E., and Ehrich, W. E., *J. Exp. Med.,* 1945, **81**, 73.

7 Dougherty, T. F., Chase, J. H., and White, A., Proc. Soc. Exp. Biol. and Med., 1944, **56**, 28; 1945, **58**, 135.

8 Philips, F. S., Hopkins, F. H., and Freeman, M. L. H., *J. Immunol.,* 1947, **55**, 289.

TABLE I.

Immunized with 5 Injections of 1 cc Horse Serum Intravenously at 2-Day Intervals, 2-3 Weeks After Adrenalectomy

Dilutions	1:1	1:25	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120
1	+++++ +++	+++++	+++++++	+++++++	+++++++	+++++++	+++++ ++	+++++ ++	+++++ ++	+++++ ++	+++ ++	++ +	++ +
2													
3													
4	+	+	+++ +	+++ +	+	+	+	+	+	+	+	+	
5	+	+	++	++	+++	+++	++	++	++	++	+	+	+
6		+		+	+	+	+	+	+	+			
7										+			

Intact Rabbits Given 5 Injections of 1 cc Horse Serum Intravenously at 2-Day Intervals.

Dilutions	1:1	1:25	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120
8	±|±|±|++	++	±|±|++|	±|+++±	++ +	+ +	++ ±|++	++ ±| +	++				
9		++	++	+++++	+++ +	+++	++ ±|++	++ ±| +	++				
10													
11													
12													

one control serum was positive in dilution of 1:2560 and in the others there was no reaction above 1:320. Far more striking than the difference between the two groups shown by dilution, is the difference in the volume of the precipitates in the lower dilution (Fig. 1).

Exp. 3. This experiment was carried out to compare the development of agglutinating antibodies in adrenalectomized and intact rabbits. A 24-hour slant culture of *Salmonella enteritidis* (mouse typhoid) was washed off with saline and the suspension heated on 3 consecutive days for 15 minutes, at 56°C. In preliminary tests it was found that while intact rabbits would withstand the intravenous inoculation of 1/10 of a culture, the adrenalectomized ones were killed by 1/1000 given intravenously and by 1/100 of a culture inoculated subcutaneously. The following method of immunization proved successful. Two adrenalectomized rabbits and two controls were each given 5 subcutaneous inoculations at 4-day intervals, starting with 1/300 of a killed culture and gradually increasing the amount up to 1/10 for the last inoculation. The animals were bled 10 days after the last injection and the sera tested for agglutinins against the organisms. The sera from the intact rabbits agglutinated up to dilutions of 1 to 40 and 1 to 80, respectively. The serum from the first adrenalectomized rabbit reacted at dilutions of 1 to 640 and the other was definite at 1 to 1280, the highest dilution used.

Effect of Adrenal Cortical Hormones on Antibody Formation in Adrenalectomized Rabbits. The marked over-development of the lymphoid system which follows removal of the adrenals may be prevented by the administration of adrenal cortical hormones. In the following experiment, the effect of suppressing the lymphoid development by the hormone treatment was tested on the antibody formation.

Two adrenalectomized rabbits were given 23 daily injections of 1 cc each of adrenal cortex hormones in oil, which was equivalent to 40 rat units or 2 mg of 14-oxy-11-dehydrocorticosterone (Upjohn Co.). This covered the period following the operation and extended through the period of immunization
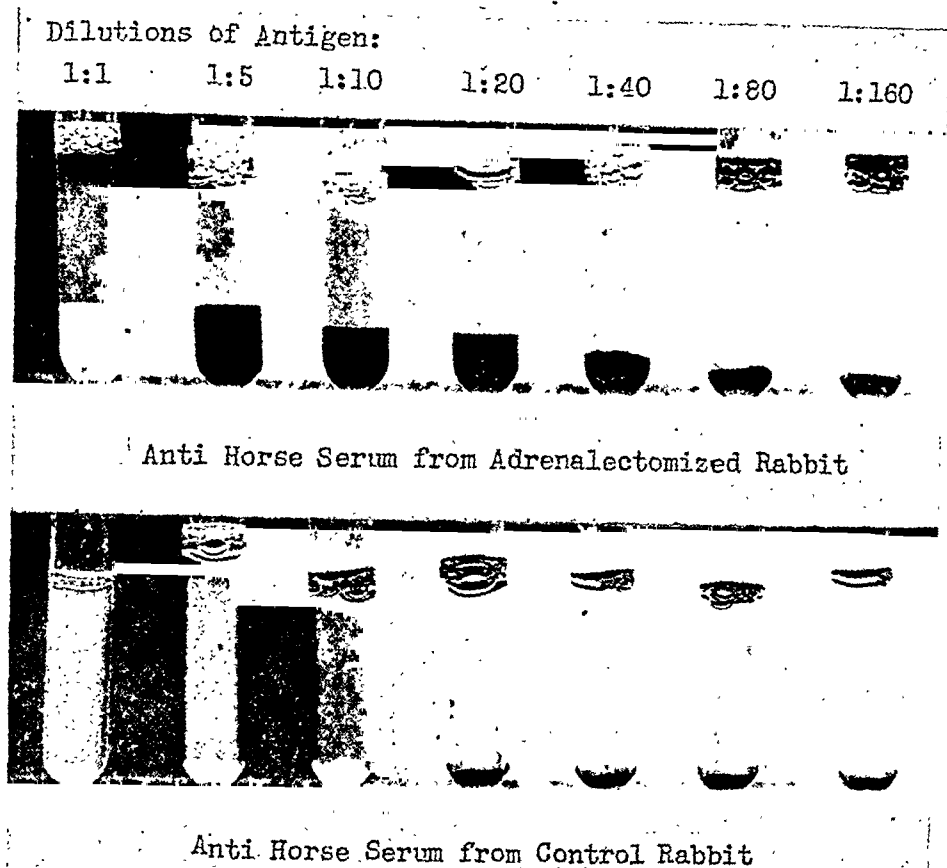
FIG. 1.

with horse serum. A third rabbit was given daily doses of one cc of Eschatin (Parke Davis and Co.). Each cc of this preparation is equivalent to 50 dog units. These animals with 2 untreated adrenalectomized rabbits and 2 intact controls were immunized with horse serum as in the previous experiment. The test of the sera showed no material difference in precipitin formation in hormone treated and untreated adrenalectomized rabbits. The intact controls gave far less response as in the preceding test.

Discussion. Ehrich and Harris[5,6] in a series of papers have adequately discussed the evidence that the lymphocyte is a factor in antibody formation. The experiments reported above are essentially a confirmation of our earlier observation,[2] that animals with hypertrophied lymphoid systems have an enhanced ability to produce antibodies. In the earlier

tests dry heat was used as the stimulus; in the present study advantage was taken of the well-known fact that extensive overdevelopment of the entire lymphoid tissue follows removal of the adrenals. If the immune substances are present in the cytoplasm of the lymphocyte and there is evidence for this belief, the release into the circulation is probably due to shedding, which is known to occur as the lymphocyte matures. One of our tests confirms the observation of Dougherty, Chase and White[7] that the release of antibodies may be definitely augmented by adrenal cortical hormones. The present experiments demonstrate that the hormone is not essential for this release and therefore, strictly speaking, antibody production is at least not entirely under the control of the adrenals.

Summary. Adrenalectomized rabbits with hypertrophy of the lymphoid tissues produce

antibodies in amounts far in excess of that produced by intact animals. This is evident not only by definite reactions in higher dilution but by the more massive precipitate in low dilutions. The prevention of the lymphoid hypertrophy by administration of adrenal cortical hormones does not reduce the amount of antibodies in the sera. This is probably due to the disruptive action of the hormone on the lymphocyte with a more rapid release of the immune globulin than would normally take place.

16073

Prevention of Experimental Arteritis in Dogs by Vitamin E.*

RUSSELL L. HOLMAN.

From the Department of Pathology, School of Medicine, Louisiana State University, and the Charity Hospital of Louisiana, New Orleans.

In a recent publication[1] data were presented to show that the combination of a specified high fat diet and experimentally induced renal insufficiency in dogs is regularly followed by predictable lesions in the arterial system. The lesions have been found in elastic arteries, in muscular arteries, and in arterioles in practically every organ and tissue of the body with the exception of the kidney and liver. Essentially the lesion is an acute necrotizing arteritis whose closest human counterparts are periarteritis nodosa and rheumatic arteritis.

When it was found that the dietary factor was lipid in nature, the question naturally arose as to the effect of vitamin E—the so-called "fat anti-oxidant" vitamin—on these experimental arterial lesions.

Methods. The methods have been detailed in previous publications.[1] Briefly these consist of feeding selected table scrap to which is added each day 3.0 cc/kg of a commercial grade of cod liver oil. After this diet has been consumed for 8 weeks or longer, the kidneys are severely damaged by any one of several methods (heavy metal injury, bilateral nephrectomy, *Leptospira Canicola* — usually

uranium nitrate, 10.0 mg/kg injected subcutaneously) and the arterial system is examined both grossly and histologically when the dogs die or are sacrificed days to weeks later.

In the first series of 4 dogs vitamin E—one capsule containing 20 mg of mixed natural tocopherols—was given by mouth between 8 and 10 o'clock in the morning and the dogs were fed the diet of table scrap and cod liver oil around 4 o'clock in the afternoon. Following kidney damage the dogs usually quit eating on the fourth to seventh day. Administration of vitamin E capsules was continued until the dog died.

In the second series of 4 dogs vitamin E (in the same form as above) was not started until *after* the kidneys were damaged. The dogs were fed table scrap and cod liver oil for 8 weeks or longer, then a lethal dose of uranium nitrate was injected and a single capsule of 20 mg mixed natural tocopherols per day by mouth was started 0, 24, 48, and 72 hours after the heavy metal injury. Vitamin E therapy was continued until the dogs died in uremia.

In these 8 dogs the single capsule of 20 mg of mixed natural tocopherols amounted to 2.5 to 4.0 mg/kg/day.

Experimental Observations. The significant data on the 4 dogs fed vitamin E concomitantly with the specified high fat diet for 8 weeks or longer before kidney damage was

TABLE I.
Effect of Vitamin E on Incidence of "Experimental" Arterial Lesions.

Group	Dog No.	Body wt at time of renal injury, kg	Period on high fat diet before renal injury, weeks	Day following renal injury on which Vit. E was started	Survival interval following renal injury, days	Arterial lesions
A Vit. E—fed concomitantly with high fat diet	45-84	5.8	8	—56	10	—
	45-85	5.9	9	—63	11	—
	45-86	7.5	10	—70	12	—
	45-87	9.4	12	—84	13	—
B Vit. E—Started after renal injury.	46-8	19.0	14	0	10	—
	46-9	13.7	14	1	8	—
	46-13	13.0	14	2	30	—
	46-12	13.7	14	3	20	+

* The grading of the lesions here is the same as that employed in previous publications, namely,

++++ = gross lesion greater than 1 cm in maximum dimension;
+++ = gross lesion less than 1 cm in maximum dimension;
++ = lesion discovered in routine histological section;
— = negative.

induced are given in Table I, section A; and section B of this table gives the same data for the 4 dogs in which vitamin E was started 0, 1, 2, and 3 days *after* renal insufficiency was produced. There is a striking difference in the incidence of arterial lesions in these two groups as compared with similar experiments in which vitamin E was not administered (Table II).

Discussion. Since the pathogenesis of the arterial lesions related to high fat diet and renal insufficiency is not understood, there is no ready explanation for the protective action of vitamin E. The problem might be approached indirectly. If we assume that vitamin E exerts its usual "anti-oxidant" action, we can postulate pathogenesis as follows: During the 8 weeks or more of dietary feeding, one or more of the "toxic" substances (fatty acids are under suspicion) contained in the diet saturates the tissues with the excess spilling over in the urine. When this safety valve is destroyed, the excess piles up to "explosive" levels and the arterial lesions ensue.

If our present theory that the toxic substance is fatty acid proves correct, a corollary of it would be that the fatty acid damages collagen, for the first detectable anatomical change is edema, swelling, fragmentation and necrosis of collagen. The subsequent fibrin, "fibrinoid" and intense leukocytic reactions that are seen in the typical arterial lesion presumably represent the response of the body to the necrotic collagen. Why the assumed fatty acid selects collagen in certain sites of the body for this activity—also whether it acts directly on the collagen in these sites or does so by inactivating vitamin C (which it has been shown to do in aqueous solutions *in vitro*[2]) are not known. Attempts thus far to demonstrate any variations in ketone bodies in the urine or any consistent variations in the blood plasma lipids at different stages in the experimental procedures have not yielded definite confirmatory evidence for the above hypothesis.

[2] Strohecker, R., and Buchholz, E., *Chem. Abstr.*, 1943, **37**, 5763.

TABLE II.
Effect of Vitamin E on Incidence of "Experimental" Arterial Lesions.

Diet	Weeks*	No. with lesions	
		No. in group	% positive
Table scrap + C.L.O.†	8-15	22/25	88.0
Table scrap + C.L.O. + Vitamin E	8-14	1/8	12.5

* Weeks of dietary feeding before production of renal insufficiency.
† C.L.O. = Cod Liver Oil.

Summary. 1. Twenty-two of 25 dogs fed a kennel diet of selected table scraps to which was added each day for 8 weeks or longer 3.0 cc/kg of cod liver oil developed typical arterial lesions when they died in uremia 7 to 35 days after the experimental production of renal insufficiency.

2. When vitamin E (mixed natural tocopherols. 2.5 to 4.0 mg/kg/day by mouth) was administered along with the diet, none of 4 dogs subjected to the same experimental procedures developed any arterial lesions.

3. Vitamin E was equally effective in preventing or retarding the arterial lesions when its administration by mouth was started 0, 1, and 2 days after renal insufficiency was induced.

16074

Cataract Development in Animals with Delayed Supplementation of Tryptophane.

A. J. SCHAEFFER* AND E. GEIGER.

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In several recent papers[1-4] demonstrating the production of lesions in the eyes of experimental animals on inadequate diets, corneal vascularization was encountered more frequently and as a more general response to deficiencies than cataract formation. The latter lesion was observed only rarely, chiefly in rats fed on tryptophane or histidine deficient diets. The development of cataract might be a result of disturbed protein synthesis due to the absence of the essential amino acids; however, since cataract did not develop in animals on diets deficient in other essential amino acids such as lysine, leucine, threonine, valine, isoleucine, arginine, etc., the possibility of a specific role of tryptophane and histidine in the metabolism of the lens must be considered. It was the intention of the present study to investigate this possibility by the method of delayed supplementation of tryptophane. One of the authors[5] has shown that infantile rats, on a diet of incomplete amino acid mixtures, did not grow if the missing amino acid was fed separate from the rest of the diet several hours later. These experiments indicated that amino acids are not stored in the body, and that protein synthesis can proceed only when all essential amino acids are simultaneously present in the tissues.

If the delayed supplementation of tryptophane would prevent cataract formation with-

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1 Chan. T. T., Chang, C. Y., and Luo, T. H., *Chinese J. Physiol.*, 1941, 16, 241.

2 Totter. R. J. and Day, P. L., *J. Nutrition*, 1942, 24, 159.

3 Albanese. A. A., and Buschke, W., *Science*, 1942, 95, 584.

4 Sydenstricker, V. P., Schmidt. H. L., Jr., and Hall, W. K., PROC. SOC. EXP. BIOL. AND MED., 1947, 64, 59.

5 Geiger. E., *J. Nutrition*, 1947, 34, 97.

out promoting growth, we might conclude that this amino acid exerts its protective action independent of protein synthesis. Such a possibility has been suggested by Cahill and Kohalik[6] who found that abrine (1-methyl-tryptophane), which is not as efficient as tryptophane in promoting growth, nevertheless prevents cataract in animals on tryptophane free diets.

The experiments were performed on infant rats (35-45 g body weight) in 2 groups of 6 each. The first group was fed the tryptophane deficient Diet I for 12 hours, and Diet II, containing tryptophane, for the next 12 hours. The second group received Diet I for 2 hours and Diet II for 2 hours; all food was withdrawn for the duration of one hour when diets were changed in this group. For further technical details see Geiger.[5]

Composition of Diet I — 30 g acidhydrolysed fish protein—70 g basal diet.

Composition of Diet II — 6 g 1(−) tryptophane — 94 g basal diet.

The basal diet contained: corn starch, 3050 g (78.3%); rice bran concentrate, 400 g (10.26%): cottonseed oil, 200 g (5.13%); U.S.P. salt mixture, 200 g (5.13%); fish oil (one g contains 2,000 I.U. vitamin A and 400 I.U. vitamin D) 50 g (1.27%); riboflavin, 75 mg; Ca pantothenate, 150 mg; choline chloride, 2.5 g.

The results regarding growth were essentially the same as those reported in the previous study. In spite of an adequate supply of tryptophane derived from the two diets consumed in 24 hours, the rats failed to show normal growth; this again demonstrated that tryptophane fed apart from the other amino acids could not be utilized for protein synthesis. We did not observe the alopecia or hyperexcitability described by Totter and Day.[2] These symptoms probably resulted from other nutritional lack (deficiency or certain vitamins, perhaps) of the diet employed by these authors.

The ocular findings were as follows. Lenticular involvement and corneal vascularisation was noted in 10 of the 12 experimental

animals. The 2 animals with no sign of cataract had been on the experimental diet for only 6 weeks. In all cases the lenticular opacities showed the character of a posterior cortical cataract. The opacities were first noted subcapsularly, but gradually involved most of the posterior cortex, and in 2 cases progressed to the anterior cortex, the nucleus, and finally the entire lens.

The shape of the opacities showed considerable variation. In one case, it involved the central portion of the posterior cortex, forming a flower shaped figure, while the peripheral region remained clear. In other cases, the peripheral margin developed denser opacities, while the central area remained clear. The suture lines became visible; dehiscense of the lines, fluid clefts and vacuoles became more and more noticeable. In a few cases the anterior cortex became involved, principally in the equatorial region. In 2 cases, this development was followed by progressive intensification of the milky, diffusely opaque appearance of the whole lens.

The period of development also showed marked variations. In most cases, some definite sign of cataract formation could be observed by the sixth week of the experiment. In the 2 cases where cataract development proceeded furthest, some incipient signs had been noted by the fourth week of the experiment with a rapid subsequent course. By the twelfth week the lenses appeared whitish and opaque to the unaided eye in these animals, while in other animals only posterior cortical opacities were observed.

These results are similar to, if not identical with, the growth and eye manifestations noted in the studies of rats on tryptophane deficient diets. There are no clinical differences between animals completely deprived of tryptophane and those receiving delayed supplementation with respect to corneal vascularisation and cataract formation.

In these experiments, ocular symptoms developed in spite of the fact that the rats ingested relatively large quantities of tryptophane (0.1 to 0.2 g per day). Prevention of cataract, like promotion of growth, can be achieved only if tryptophane is supplied

6 Cahill, W. M., and Kotalik, G. C., J. Nutrition, 1943, 26, 471.

simultaneously with the other essential amino acids. This suggests a possible causal relationship between disturbed protein synthesis and cataract formation. Further investigations as to why disturbance of protein synthesis by the lack of essential amino acids other than tryptophane and histidine does not result in cataract formation is required.

Summary. Experiments with delayed supplementation of tryptophane show that cataract formation in rats occurs even after consumption of relatively large quantities of tryptophane. It seems that like growth promotion. cataract prevention by this amino acid can be accomplished only when it is supplied simultaneous with the other essential amino acids.

16075

Simulated Auricular Flutter in the Electrocardiogram of the Dog.

A. SURTSHIN. (Introduced by L. N. Katz.)

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In the course of studies with epinephrine in the unanesthetized dog, undulations of the baseline at a rate of the order seen in auricular flutter developed in the electrocardiographic tracing. At first sight, the mechanism appeared to be auricular flutter. The coincident occurrence of tachypnea and retching during the recording of these electrical phenomena suggested the possibility that these undulatory waves might be extracardiac, caused by retching and tachypnea.

The possibility exists that similar records in man might, on occasion, be misinterpreted as auricular flutter. It thus appears advisable to present these records for publication.

In Fig. 1A are shown two sections of a continuous recording of lead 2 obtained in the dog after the intravenous injection of one mg epinephrine. In the first few cardiac cycles of the top strip regular undulations in the baseline at a rate of 356 per minute resemble the "F" waves of auricular flutter. This was associated with tachypnea of the same rate. The ventricular rate is 65 per minute. In the middle of the tracing during a period of transitory apnea, the undulations are absent, no P waves are seen, and the contour and rate of the ventricular complexes are unchanged (the QRS and T waves are huge and upright). Toward the end of the top strip the phenomenon in the first part of the tracing recurs and continues during the first part of the second strip which was recorded 2 minutes later. In the middle of the second strip it is possible to identify P waves even though they are partially obscured by the undulations. Some of these are labeled. The ventricular rate at this time is 88 per minute and irregular. We are dealing here with a nodal rhythm followed by a sinus arrhythmia at a faster rate and the record is obscured by extracardiac artefacts simulating impure auricular flutter.

Fig. 1B is a tracing of the femoral arterial pressure in the same dog recorded with a Hamilton manometer simultaneously with a pneumogram obtained with a pneumograph strapped around the animal's chest. Below is a baseline recording time in seconds. The pneumogram shows post-epinephrine tachypnea at a rate of 306 per minute. In some instances, not seen here, these respiratory waves may even appear as noticeable fluctuations of the blood pressure within a single cardiac cycle.

Fig. 2 is a continuous electrocardiographic tracing (lead 2) obtained under similar circumstances in another dog. At the beginning
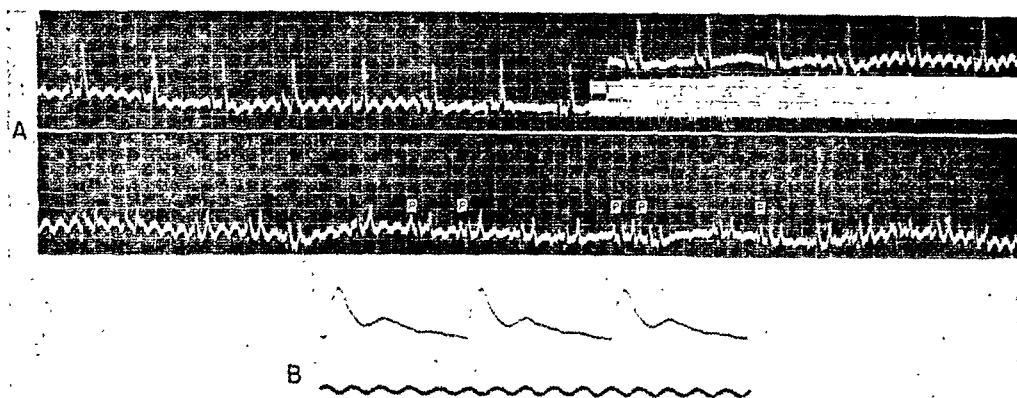
FIG. 1.

FIG. 2.

of the record ventricular tachycardia with a rate of 150 per minute is present with upright and prolonged QRS complexes (going off the record) and with deeply inverted T waves. Undulations of the baseline with a rate of 333 per minute begin in the middle third of the record and continue to the middle of the second strip. At times, they attain a huge size. When these undulations first appear, the ventricular rate slows abruptly to about 65 per minute, the configuration of the ventricular complexes changes, and the QRS becomes shorter in duration. No P waves are seen even at the end of the second strip when the undulations have disappeared. The baseline undulations were observed to have occurred when the dog began to retch and vomit and are therefore probably attributable to artefacts caused by diaphragmatic movements (extracardiac artefacts). It appears that the ventricular tachycardia present at the beginning of the record broke at the on. of retching and was followed by A-V nodal rhythm.

Conclusions. Baseline movements with simulated impure auricular flutter developed following intravenous epinephrine injections. These are shown to be extracardiac in nature. They are induced by extreme tachypnea and the diaphragmatic movements associated with retching and vomiting.

16076

Influence of Pamaquine and Atabrine on the Enzymatic Degradation of Quinine.

Graham Chen.*†

From the Department of Pharmacology, University of Chicago, Chicago, Ill.

The purpose of the present investigation is (1) to devise a procedure for the quantitative evaluation of the activity of the enzyme which destroys quinine and (2) to study the effect of pamaquine and atabrine on the degradation of quinine by the enzyme.

Materials and Methods. Suspensions of fresh rabbit's liver were prepared by homogenization with Ringer-Locke solution in a Waring blender. They were centrifuged at 1200 r.p.m. for 15 minutes; the supernatant only was used in the following experiments. The mixture and the supernatant were found to give the same enzyme activity and the same relationship between concentration and effect. However, more consistent results were obtained with the latter.

The enzymatic digestion was conducted at 39°C in an incubator. The enzyme preparation and the quinine solution of Ringer-Locke were kept in an incubator for half an hour before mixing. As a rule, 0.5 ml of the former was pipetted into 3.5 ml of the latter in a 30 ml centrifuge tube. At appropriate times equal volume of 4% NaOH was added to the digestion medium to terminate the reaction.

The determination of quinine was carried out by a method which has previously been described.[1] In brief, quinine was extracted from the digestion medium at an alkalinity of 2% NaOH with ethylene dichloride. In control experiments, with and without heating on a steam bath for an hour, the amount of quinine extracted with ethylene dichloride at this concentration of NaOH was found to be the same. The concentration of quinine was measured fluorometrically with the Coleman

photofluorometer (model 12). Pamaquine does not give any fluorometric reading with B_1, PC_1 filters. Atabrine shows a slight fluorescence through these filters; it does not, however, interfere with quinine determination. A blank value for atabrine is subtracted from the total fluorometric readings when quinine is determined in the presence of atabrine.

Results and Discussion. The graphs in Fig. 1 indicate the relationships between the percentage degradation of quinine and the concentration of liver. The rate of the reaction is shown by curves in Fig. 2. Except for low concentrations and during the beginning of the experiment, the degradation of quinine is in a linear relationship with the concentration of liver or with the time of reaction in logarithms. The linearity in rate was found to
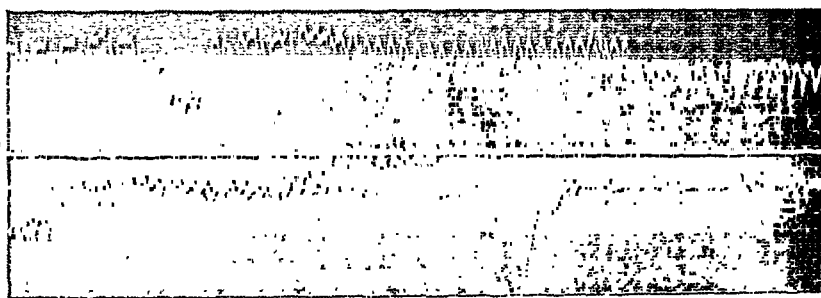
Fig. 1.

FIG. 1.

FIG. 2.

of the record ventricular tachycardia with a rate of 150 per minute is present with upright and prolonged QRS complexes (going off the record) and with deeply inverted T waves. Undulations of the baseline with a rate of 333 per minute begin in the middle third of the record and continue to the middle of the second strip. At times, they attain a huge size. When these undulations first appear, the ventricular rate slows abruptly to about 65 per minute, the configuration of the ventricular complexes changes, and the QRS becomes shorter in duration. No P waves are seen even at the end of the second strip when the undulations have disappeared. The baseline undulations were observed to have occurred when the dog began to retch and vomit and are therefore probably attributable to artefacts caused by diaphragmatic movements (extracardiac artefacts). It appears that the ventricular tachycardia present at the beginning of the record broke at the o... ' of retching and was followed by A-V nodal ; rhm.

Conclusions. Baseline movements with simulated impure auricular flutter developed following intravenous epinephrine injections. These are shown to be extracardiac in nature. They are induced by extreme tachypnea and the diaphragmatic movements associated with retching and vomiting.

16077

Urinary Excretion of Amino Acids by the Rat.*

B. S. SCHWEIGERT. (Introduced by P. B. Pearson.)

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The adaptation of microbiological methods for the determination of amino acids in urine[1-8] has afforded considerable information on the levels of various amino acids excreted by several species as influenced by the amounts ingested. A paucity of information is available, however, on the influence of dietary deficiencies, such as specific amino acid or vitamin deficiencies, on the excretion of amino acids in the urine. In earlier work[2,9] it was shown that vitamin B_6 deficient rats metabolized tryptophane to microbiologically inactive products at a rate comparable to that observed for vitamin B_6 supplemented animals. In the present study the effect of the ingestion of tryptophane deficient diets and the ingestion of diets deficient in riboflavin or nicotinic acid on the amounts of certain amino acids excreted in the urine was determined.

Experimental. Care of animals and composition of diets. Weanling rats were housed in single cages and were fed the experimental diets for varying periods of time. Variations in the composition of the diets used were either in the level or kind of protein or in the B vitamin supplement added. Corn oil was added to all rations at a level of 4.7%, vitamin A and D concentrate 0.3%, salt mixture 4.0% and sucrose to 100%. Unless indicated otherwise, each diet contained 250 μg each of thiamin and pyridoxine, 300 μg of riboflavin, 2 mg of calcium pantothenate, 100 mg each of choline and inositol and one mg of nicotinic acid per 100 g. The diets were fed *ad libitum*. A brief designation of the diets is as follows:

No. 7—24% casein.
> 8—Same as No. 7 except no riboflavin was added.
> 9—12% casein.
> 10—12% casein and 12% gelatin.
> 11—Same as No. 10 except no nicotinic acid was added.
> 12—12% oxidized casein† plus adequate quantities of cystine and methionine. This ration was designed to be deficient in tryptophane.
> 13—Same as No. 12 plus 0.2% dl.tryptophane.

† The oxidized casein used in this investigation was prepared by the method of Toennies, *J. Biol. Chem.*, 1942, **145**, 667.

Urine collections were made by the use of metabolism cages fitted with outside feeders. The urine was collected during a 2- or 3-day period and food consumption records were also kept for these periods. Collections were made at least twice during the experimental period and data on at least two animals were obtained for each series of collections. Thus data on the amino acids excreted were obtained at different times throughout the experimental period. The urine samples from each group were combined prior to analysis so that data were obtained on 4 or more rats in each group. The collections were made at the same time for each series of deficient and sup-

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* Frances Panzer and Helen Keene assisted with the amino acid analyses.

[1] Schweigert, B. S., Sauberlich, H. E., and Elvehjem, C. A., *Science*, 1945, **102**, 275.

[2] Schweigert, B. S., Sauberlich, H. E., Elvehjem, C. A., and Baumann, C. A., *J. Biol. Chem.*, 1946, **164**, 213.

[3] Sauberlich, H. E., and Baumann, C. A., *J. Biol. Chem.*, 1946, **166**, 417.

[4] Steele, B., Sauberlich, H. E., Reynolds, M., and Baumann, C. A., *J. Nutrition*, 1947, **33**, 209.

[5] Harvey, C. C., and Horwitt, M. K., *Fed. Proc.*, 1947, **6**, 259.

[6] Hier, S. W., and Bergeim, O., *Fed. Proc.*, 1947, **6**, 261.

[7] Frankl, W., and Dunn, M. S., *Arch. Biochem.*, 1947, **13**, 93.

[8] Dunn, M. S., Camien, M. N., Shankman, S., and Block, H., *Arch. Biochem.*, 1947, **13**, 207.

[9] Schweigert, B. S., and Pearson, P. B., *J. Biol. Chem.*, 1947, **168**, 555.

hold as long as 8 hours for a digestion medium containing 5 μg/ml of quinine and 0.25% of liver. Whether or not this relationship holds true for the pure enzyme and quinine awaits confirmation. If so, it may reveal the reaction mechanism of the enzymatic process.
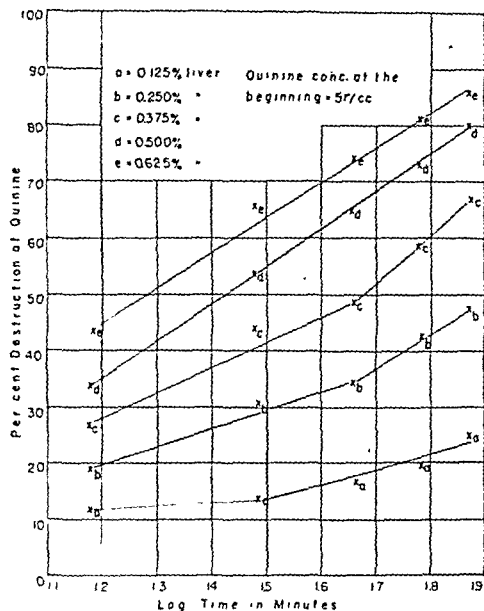

FIG. 2.
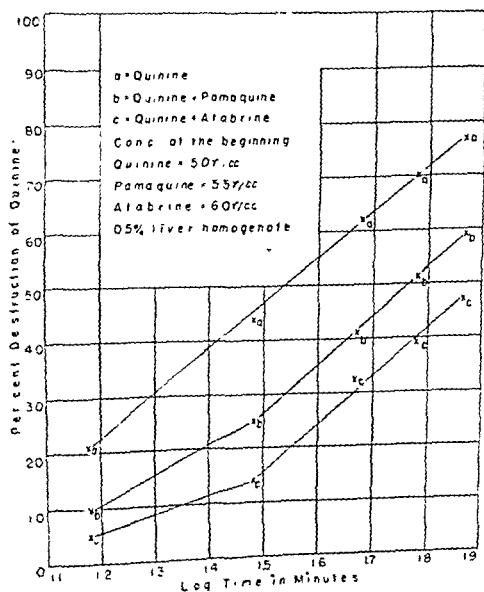
FIG. 3.

Clearly the linear relationship between concentration and effect for a certain duration of reaction, as shown in Fig. 1, may be utilized to determine quantitatively the enzyme activity of a preparation. The duration of one hour was chosen in the present work. The relationship deviates from linear at shorter intervals. The line for one hour will be taken as a reference standard in subsequent calculations for the enzyme activity of a sample by locating the corresponding value on the abscissa to the per cent degradation of quinine on the ordinate. For convenience in calculations, the value one on the abscissa will be considered as 10 units of enzyme activity.

The effect of pamaquine and the effect of atabrine on the enzymatic destruction of quinine were carried out under identical conditions as for the experiment in obtaining the reference standard; *i.e.* in a medium containing the supernatant of a 0.5% liver homogenate, 5 μg per ml of quinine and a molecular equivalent quantity of pamaquine or atabrine. Data in Fig. 3, obtained with the same sample of liver homogenate, indicate that the enzymatic destruction of quinine was decreased by the addition of pamaquine or atabrine. There is, however, no difference in the rate of the reaction. By interpolation from the line for one hour in Fig. 1, the enzyme activities as represented by the curves in Fig. 3 for the same period are as follows: for quinine alone, 50.1 units; quinine plus pamaquine, 30.2 units and for quinine plus atabrine, 22.4 units. The suppression of enzyme activity by pamaquine or atabrine is thus 39 and 54% respectively. It is not understood whether the effect of these two compounds is on the enzyme or on the substrate in the enzyme system. As judged by the rate of the reactions with and without pamaquine or atabrine, the effect of the two compounds is probably on the enzyme.

Summary. A procedure has been devised for the quantitative evaluation of the activity of the enzyme which degradates quinine. Pamaquine and atabrine were found to suppress the enzymatic degradation of quinine by a liver homogenate.

plemented groups. The urine samples were stored in a refrigerator prior to analysis.

Amino acid analyses. Histidine, arginine, threonine, phenylalanine and tryptophane determinations were made after neutralization and proper dilution of the urine samples. These amino acids were chosen for analysis since it seemed likely that amino acids of this type may be more difficult to metabolize than the simpler amino acids. In addition, the methods for determining these amino acids in urine are quite accurate while the reliability for others is somewhat uncertain. *S. faccalis* R was used as the test organism for the determinations of threonine, arginine and histidine. The medium used has been described previously.[10] Tryptophane and phenylalanine were determined with *L. arabinosus* as the test organism.[1,11]

In some cases the occurrence of bound forms of the amino acids was investigated. Aliquots of the urine samples were hydrolyzed with one N HCl at 15 pounds pressure for 5 hours, neutralized, diluted to an appropriate volume and assayed for amino acids. By this procedure, data were obtained on the amounts of certain amino acids that were excreted in combined form, since the same samples were analyzed for amino acids prior to hydrolysis.

Results and Discussion. The amounts of histidine, arginine, threonine, phenylalanine and tryptophane excreted per day and the percentage of each amino acid ingested that was excreted in the urine are shown in Tables I and II. The latter figures were computed on the basis of the amount of each amino acid consumed and of the amount of each amino acid excreted per day. The composition accepted for casein was: histidine 2.6%, arginine 3.6%, threonine 4.0%, phenylalanine 4.7% and tryptophane 1.2%. For gelatin the values used were 0.6% histidine, 7.8% arginine, 1.8% threonine, 2.0% phenylalanine and 0.2% tryptophane. The amounts in casein and gelatin were determined by

microbiological methods. From these data and from the food consumption data the amounts of each amino acid ingested per day were calculated.

With the methods employed, the values for urine shown in Tables I and II represent the amount of each amino acid present in the urine that is microbiologically available. These values have also been termed the amount of apparent free amino acid present. Undoubtedly, the free amino acids comprise the largest portion measured, but it is recognized that certain amino acids present as peptides are utilized to some extent by the test microorganisms.[12] In order to determine the total amount present, it is necessary to hydrolyze the urine samples to liberate the bound forms of the amino acids.

It will be noted that even when rations deficient in an amino acid or a vitamin are fed for considerable periods of time, the amounts of the amino acids excreted per day are not materially different than those excreted by the control animals. Similarly, no difference was observed in the amounts excreted when determinations were made at different times in the experimental period. The percentage of the ingested amino acids excreted in the urine was somewhat higher, however, for the tryptophane or riboflavin deficient groups than for the respective control groups. The differences in the percentages of the ingested amino acids excreted were not as consistent, however, for the older rats fed the tryptophane deficient and supplemented diets (Table I). Rats fed a nicotinic acid deficient ration excreted approximately the same percentage of these amino acids as did the controls. In general, the amounts of each amino acid excreted when the control diets were fed (No. 7, 9, 10 and 13) were roughly proportional to the amino acid intake. The amount of arginine excreted, for example, was much higher when ration 10 (12% casein + 12% gelatin) was fed than when ration 9 (12% casein) or ration 7 (24% casein) was fed. This is undoubtedly due to the higher amounts of arginine ingested when ration 10

10 Greenhut. I. T., Schweigert, B. S., and Elvehjem, C. A., *J. Biol. Chem.*, 1946, 162, 69.

11 Schweigert. B. S., McIntire, J. M., Elvehjem, C. A., and Strong, F. M., *J. Biol. Chem.*, 1944, 155, 183.

12 Schweigert, B. S., and Snell, E. E., *Nutr. Abs. and Rev.*, 1947, 16, 497.

TABLE I.
Effect of Feeding Diets Low in Tryptophane on Urinary Excretion of Certain Amino Acids.*

Diet No. and description	Time on experiment, days	Wt change, per day, g	Histidine Amt excreted daily, mg	Histidine Ingested amino acid excreted, %	Arginine Amt excreted daily, mg	Arginine Ingested amino acid excreted, %	Threonine Amt excreted daily, mg	Threonine Ingested amino acid excreted, %	Phenylalanine Amt excreted daily, mg	Phenylalanine Ingested amino acid excreted, %	Tryptophane Amt excreted daily, mg	Tryptophane Ingested amino acid excreted, %
No. 12 (12% oxid. casein basal)	7-14	—0.9	0.15	2.1	0.28	2.3	0.17	1.4				
No. 13 (12% oxid. casein + tryptophane)												
No. 12 (Group 2)†	7-14	+2.2	0.13	0.95	0.16	0.73	0.14	0.64	0.14	0.50	0.037	—
No. 13 (Group 2)†	4-10	—1.5	0.22	1.4	0.24	1.0	0.36	1.5	0.59	0.89	—	—
No. 13 (Group 2)†	4-10	+1.0	0.36	0.98	0.56	1.0	0.43	0.76				
No. 7 (24% casein)	14-26	+4.4	0.25	0.34	0.64	0.57	0.54	0.48	0.36	0.27	0.15	0.41
No. 9 (12% casein)	14-26	+3.5	0.22	0.59	0.58	1.0	0.30	0.52	0.36	0.45	0.07	0.37

* Since the amounts excreted per day were very similar for urine collections made at different times during the experimental period, the values indicated are the averages for all collections made during the experimental period. The weight change was computed for only that period during which urine collections were made.

† Rats with an initial weight of 150 g were used in these experiments.

TABLE II.
Excretion of Amino Acids by Riboflavin and Nicotinic Acid Deficient Rats.*

Diet No. and description	Time on experiment, days	Wt change, per day, g	Histidine Amt excreted daily, mg	Histidine Ingested amino acid excreted, %	Arginine Amt excreted daily, mg	Arginine Ingested amino acid excreted, %	Threonine Amt excreted daily, mg	Threonine Ingested amino acid excreted, %	Phenylalanine Amt excreted daily, mg	Phenylalanine Ingested amino acid excreted, %	Tryptophane Amt excreted daily, mg	Tryptophane Ingested amino acid excreted, %
No. 7 (riboflavin supp.)	14-54	+3.1	0.17	0.25	0.48	0.46	0.68	0.65	0.40	0.32	0.18	0.53
No. 8 (riboflavin def.)	14-54	+0.3	0.22	0.67	0.64	1.3	0.59	1.17	0.45	0.76	0.14	0.85
No. 10 (12% casein, 12% gelatin + N.A.)	14-45	+2.8	0.37	0.77	1.41	0.80	0.81	0.92	0.46	0.47	0.13	0.66
No. 11 (12% casein, 12% gelatin — N.A.)	14-45	+0.4	0.18	0.75	0.61	0.68	0.32	0.72	0.28	0.55	0.058	0.58

* Since the amounts excreted per day were very similar for urine collections made at different times during the experimental period, the values indicated are the averages for all collections made during the experimental period. The weight change was computed for only that period during which urine collections were made.

Although measurements of the amino acids in the urine and balance studies to ascertain the amount of any amino acid that is retained by the body are of great value, it should be recognized that alterations in the degree of metabolism of any amino acid are not necessarily determined by the microbiological methods used. Thus, when diets deficient in a specific amino acid or a vitamin are fed and no large differences in the excretion of the unchanged amino acid is observed, it may be quite possible that the amino acid is only partially metabolized and these partial metabolites excreted in the urine are microbiologically inactive. That marked changes in the amounts of the amino acids excreted in the urine do occur with mice fed various deficient diets has already been observed. Further studies on the specificity of these amino acid methods and their reliability with respect to blood and urine analysis will aid in obtaining more information on the metabolism of specific amino acids by various species.

Summary. Rats have been fed diets deficient in tryptophane, riboflavin or nicotinic acid and the amounts of microbiologically available histidine, arginine, threonine, phenylalanine and tryptophane excreted in the urine were determined. No large changes were noted in the amounts of these amino acids excreted in the urine when rations deficient in nicotinic acid were fed. Rats fed a riboflavin or a tryptophane deficient diet excreted approximately twice as much of the ingested amino acids as animals fed adequately supplemented diets. In all cases, however, the amounts excreted were small, less than 2.5 per cent of those ingested. After acid hydrolysis of the urine samples, a 2-4 fold increase was observed in the values for histidine, arginine, threonine and phenylalanine.

15 Albanese, A. A., Holt, L. E., Frankston, J. E., and Irby, V., *Fed. Proc.*, 1946, 5, 118.

16078

Vasoconstrction Elicited by Addition of Plasma to Vasoinactive Tissue Extracts.[*]

E. MYLON AND J. H. HELLER. (Introduced by M. C. Winternitz.)
From the Laboratory of Pathology, Yale University School of Medicine.

Perfusion experiments on the rabbit's ear have shown that several vasoinactive, renin-containing protein fractions of hog kidney became vasoconstrictive when the perfusion fluid (Ringer-Locke) was supplemented by minute amounts of epinephrine.[1]

Identical effects were observed when the kidney fractions were replaced by similarly prepared fractions of other organs—especially liver.[2]

A change from vasoinactivity to vasoconstriction in the rabbit's ear was previously reported[3] when plasma instead of Ringer-Locke was used for perfusion with kidney fractions (renin). This constriction was not attributed to the direct combined effect of the kidney fraction and plasma, but to the formation of a special vasoconstrictive substance, angiotonin (hypertensin).

Since plasma is known to contain traces of epinephrine,[4] it seemed desirable to investigate whether this epinephrine content might have contributed to the constrictive effect elicited by kidney- or other tissue-fractions.

1 Mylon, E., Horton, F. H., and Levy, R. P., PROC. SOC. EXP. BIOL. AND MED. 1947, 66, 375.

2 Mylon, E., Horton, F. H., and Levy, R. P., PROC. SOC. EXP. BIOL. AND MED., 1947, 66, 378.

3 Page, I. H., and Helmer, O. M., *J. Exp. Med.*, 1940, 71, 29.

4 Shaw, E. H., *Biochem. J.*, 1938, 32, 19.

was fed since this ration contains gelatin which is very high in arginine.

Thus it appears that the deficiencies studied do not markedly affect the ability of the rat to metabolize the ingested amino acids to microbiologically inactive products even when the animals are losing weight at a rapid rate. It is quite possible, of course, that prolonged or chronic deficiencies may cause the rat to excrete much larger quantities of these amino acids. It is also possible that the metabolism of other amino acids not studied was materially affected. Some tests conducted with lysine and methionine indicate that they also were excreted in small amounts when diets deficient in tryptophane or riboflavin were fed. Preliminary studies indicate that vitamin B_6 deficient rats do not show large changes in the amounts of these amino acids excreted. These results are quite different from those observed with the mouse[13,14] in that this species excretes large amounts of certain amino acids when deficient diets are fed. Thus an average of 37.3% of the ingested amino acids was excreted by mice when fed a tryptophane or methionine deficient diet while an average of only 2.6% was excreted when adequate diets were fed. Differences in the percentage of the ingested tryptophane that was excreted when normal diets were fed to rats as compared to mice have been discussed in earlier work.[2]

Data obtained on the amounts of histidine, arginine, threonine and phenylalanine determined after acid hydrolysis of the urine samples support the same conclusions as those on the amounts of microbiologically available amino acids (Table III); namely, that the amounts of these amino acids excreted are not appreciably affected by the ingestion of deficient diets by the rat. The results show that about ¼ to ½ of the total amount excreted is measured as the free amino acid. These results are in agreement with those reported by Sauberlich and Baumann[3] and Albanese et al.[15]

13 Sauberlich, H. E., Pearce, E. L., and Baumann, C. A., Fed. Proc., 1947, 6, 420.

14 Pearce, E. L., Sauberlich, H. E., and Baumann, C. A., J. Biol. Chem., 1947, 168, 271.

TABLE III.

Effect of Acid Hydrolysis on Amount of Certain Amino Acids Found in Urine.* (All values expressed as mg excreted per day.)

Diet No.	Dietary regimen	Histidine		Arginine		Threonine		Phenylalanine	
		before	after	before	after	before	after	before	after
7	(24% casein)	0.25	1.4	0.64	1.6	0.54	1.5	0.36	1.6
9	(12% casein)	0.22	0.72	0.58	1.3	0.30	1.0	0.36	1.1
10	(12% casein, 12% gelatin)	0.37	1.2	1.41	3.0	0.81	1.7	0.46	1.8
11	(12% casein, 12% gelatin — N.A.)	0.18	0.78	0.61	1.8	0.32	0.83	0.28	1.3
12	(12% oxid. casein — tryptophane)	0.15	0.50	0.28	0.44	0.17	0.60	—	—
13	(12% oxid. casein + tryptophane)	0.13	0.36	0.16	0.42	0.14	0.35	—	—

* The values were obtained on the same samples before and after acid hydrolysis.

FIGURE II

Plasma or minute amounts of epinephrine if combined with a vasoinactive liver fraction act almost identically.

Recent studies[4,7,8,9] indicating that the blood contains substances structurally related to epinephrine point to the possibility that these compounds are biologically active and involved in the reaction reported in this paper.

6 Bain, W. A., Gaunt, W. E., and Suffolk, S. F., *J. Physiol.*, 1937, **91**, 233.

7 Whitehorn, J. C., *J. Biol. Chem.*, 1935, **108**, 633.

8 Bloor, H. R., and Bullen, S. S., *J. Biol. Chem.*, 1941, **138**, 727.

9 Raab, H., *Biochem. J.*, 1943, **37**, 470.

Summary. Vasoconstriction of the isolated vessels of the rabbit's ear follows the addition of fresh plasma to perfusates containing liver preparations that are in themselves inactive. The activation caused by plasma is indistinguishable from the effect of subthreshold amounts of epinephrine added to these tissue fractions. Incubation of plasma, known to result in loss of its epinephrine content, also causes loss of its vasoconstrictive influence on perfusate containing liver fractions.

The following experiments were carried out to clarify this point.

Materials and Methods. Rabbit plasma was prepared as described by Landis[5] with especial care in the heparinization of the animal 5 minutes before drawing the blood. This was then spun down at $+5°C$ for 45 minutes, the plasma siphoned off and stored at the same low point until used. Fractions of liver extracts were prepared in accord with previously reported methods.[2] Epinephrine (Parke-Davis 1:1000) was freshly diluted several times a day to 1:100,000 with distilled water and used as stock solution. Further dilutions made from this concentration were used within 2 minutes. The subthreshold dose was usually between 1 cc and 1.5 cc of a 1:5,000,000 solution. The isolated rabbit's ear was chosen for perfusion. The volume of each separate substance to be tested was brought up to a constant amount by the addition of Ringer-Locke.

Results. The plasma from 5 of the 25 normal rabbits used in this study was in itself slightly and transiently vasoconstrictive on perfusion. The 25% constriction for 2 minutes observed with these 5 plasmas increased to 80% for 8 minutes on the addition of liver extract. This liver extract was completely vasoinactive if used for perfusion without plasma. Nineteen of the remaining 21 plasma samples had no effect on the isolated vessels until the liver extract was added to the perfusate. The degree of vasoconstriction recorded with these 19 samples varied from 60 to 99%, lasting for 3 to 10 minutes. The plasma samples of the two remaining rabbits elicited strong vasodilatation if added to the Ringer-Locke perfusate. Addition of the liver preparation abolished this vasodilatation.

The next experiments were designed to compare the effects of plasma and epinephrine on perfusion fluids containing liver preparations. Various combinations and sequences were tried. In none was it possible to differentiate between the effects of epinephrine and plasma on the liver containing perfusate. Typical experiments are recorded in Fig. 1 and Fig. 2.

5 Landis, E. M., Wood, J. E., Jr., and Guerrant, J. L., *Am. J. Physiol.*, 1943, **139**, 26.
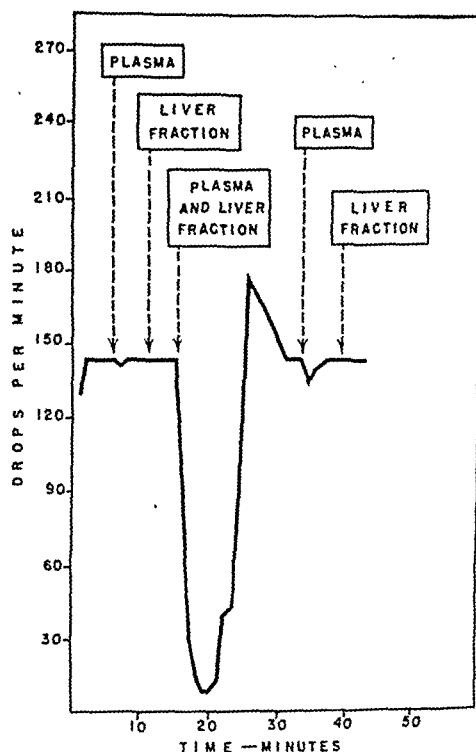
FIGURE—1
Vasoconstriction caused by the combination of vasoinactive plasma with a vasoinactive liver fraction.

All of the previous observations were carried out with fresh plasma that had been stored for not more than 3 hours at $+5°C$.

In the light of the fact that the epinephrine content of plasma is destroyed on incubation,[6] a new series of experiments was initiated in which the plasma was incubated for 12 hours before its use. The effect of this preliminary incubation on the plasma was striking: in none of the 8 experiments was any constriction obtained with plasma alone or in the combination of the incubated plasma with the liver fraction.

Discussion. The similarity of action of fresh plasma and subthreshold amounts of epinephrine suggests that the epinephrine content of the plasma is responsible for the observed effect on the rabbit's ear.

The colorimetrically determined values for "true" blood epinephrine, however, are smaller than the subthreshold doses that were used with the rabbit's ear.

TABLE I.
Deposition of liver glycogen

Group	Days after tumor implant	No. animals	Glucose, mg	Avg. body wt, gm	Liver glycogen, %	Total mg liver glycogen
Normal	—	27	0	19.6	0.29±0.02*	3.1
,,	—	33	100	20.9	2.25±0.03	26.9
Sarcoma 180	7-10	5	0	18.0	0.19±0.02	2.3
,, ,,	11-14	5	0	18.1	0.46±0.14	3.3
,, ,,	7-10	17	100	20.4	1.15±0.08	14.4
,, ,,	11-14	16	100	18.7	1.25±0.05	15.1

* Probable error of mean value.

All glycogen values are expressed in terms of glucose. Under the conditions of these experiments, the normal mice stored liver glycogen to the extent of 23.8% of the injected glucose as contrasted with only 12.0% by those animals with tumors. The probable errors of the means indicate that this is a highly significant difference whereas the difference between the mean values of the two tumor-bearing groups is not statistically significant.

Discussion. In view of the fact that, (a) the tumor-bearing mice did not lose weight; (b) their fasting liver glycogen concentrations were as high as those of normal mice; and (c) their food intake was as high as the normal, this difference can not be explained on a nutritional basis in the usual sense. Neither was the bodily activity of the tumor-bearing mice observed to be greater than that of the controls during the period between glucose injection and sacrifice. It is clear from the data that the length of time which

the mice have borne the tumors, at least between 7 and 14 days, has no influence on the glycogen content. Neither was the mass of the tumor found to influence the extent of glycogen storage. A group of animals bearing tumors having an average weight of 325 mg averaged 1.32% liver glycogen whereas a group with 1,200 mg tumors were found to average 1.24%. Greenstein[4] found that the decrease in liver arginase and catalase activities of animals bearing tumors were likewise independent of the age of the tumor. It is possible that the impaired glycogenesis may result from a similar effect on the enzyme systems in the livers.

Conclusion. Male mice bearing sarcoma 180 implants exhibit an impairment in their ability to store liver glycogen after glucose administration. This is similar to the defect in patient with gastric cancer.

[4] Greenstein, J. P., *J. Nat. Cancer Inst.*, 1942-1943, 3, 419.

16080

Arginase and Catalase Activity in Livers of Patients Having Benign and Malignant Gastric Lesions.*

N. F. YOUNG, VERA COLLIER AND F. HOMBURGER. (Introduced by C. P. Rhoads.)
From the Department of Clinical Investigation, the Sloan-Kettering Institute for Cancer Research, New York City.

The comparison of activities of certain

enzymes of normal adult tissues with those of tissues undergoing rapid proliferation has revealed differences. These have given rise to much speculation regarding the possible relationship of enzyme activity to neoplastic growth. One important phase of this work

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Deposition of Liver Glycogen in Normal Mice and in Mice Bearing Sarcoma 180.*

N. F. YOUNG, C. J. KENSLER, LOUISE SEKI, AND F. HOMBURGER.
(Introduced by C. P. Rhoads.)

From the Laboratory of Clinical Investigation, the Sloan-Kettering Institute for Cancer Research, New York City.

Previous investigation in this laboratory has demonstrated an impairment in the ability of patients bearing gastric cancer to lay down liver glycogen after the oral administration of glucose.[1] If small laboratory animals can be shown to be similarly affected by the presence of a tumor, more extensive and detailed studies of the abnormality would be possible. In animals the factors which normally influence glycogen storage can be better controlled. Moreover, the influence of tumor type and size as well as other variables pertinent to the study can be investigated more easily than is possible in human subjects.

This paper describes the results of measurements of liver glycogen in normal mice and in mice bearing sarcoma 180 implants before and after glucose administration.

Methods. Young, adult male mice, CFW strain, which were obtained from Carworth Farms, and which weighed from 16 to 26 g were used in all experiments. All animals were allowed drinking water *ad libitum* and were placed on a diet of Purina Laboratory Pellets for at least 2 days, to eliminate gross differences in nutritional state before use.

These animals were divided into 4 experimental groups. Two groups had had sarcoma 180 implanted into the axillary region 7 to 14 days previous to the experiment. Of these animals, one group provided fasting liver glycogen levels while the other group was treated with glucose before liver glycogen assay was made. The other 2 groups consisted of normal mice. One group was fasted and one was given glucose. All animals were weighed frequently and on some occasions the food intake was followed. Except for those animals which had borne tumors for 12 to 14 days, all appeared healthy.

At 4 p. m. on the day preceding each experiment, the mice were deprived of food and placed in clean cages in groups of 2 to 4 in a dark, quiet, air-conditioned room.[2] After 16 hours, they were either sacrificed for the determination of fasting glycogen levels or were given an intraperitoneal injection of 100 mg of glucose (0.25 ml of 40% glucose in water) and sacrificed 2 hours later to allow measurement of the increase in liver glycogen over the control value. The mice were killed by severing the spinal cord at the base of the brain to minimize struggle. The whole liver was removed, weighed, and dropped into 30% KOH within 60 seconds of the time of death. Liver glycogen was determined by the Good, Kramer and Somogyi[3] modification of Pfluger's method.

Results. As can be seen in Table I, the 16-hour fast sufficed to reduce the liver glycogen levels to a uniformly low level in both normal and sarcoma-bearing animals. The difference between the fasting levels in the groups of animals tested after bearing tumors for 7-10 days and 10-14 days is probably due to the small numbers of animals used and in any case is not significant in so far as this study is concerned, since these experiments were not designed to demonstrate differences in rates of glycogen depletion.

1 Abels, J. C., Young, N. F., and Homburger, F., in preparation.

2 Swensson, Ake. *Acta Phys. Scand. Supp.,* 1945, 2, 33.

3 Good, C. A., Kramer, H., and Somogyi, M., *J. B. C.,* 1933, 100, 485.

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TABLE I.
Arginase and Catalase Activities of Human Liver Biopsies.

No.*	Sex	Age	Diagnosis	Catalase ml O_2/MgN/sec	Arginase AU/mgN†
82506	m	55	Gastric Ulcer		1.00	9.1
49722	m	64	,, ,,	0.96	8.3
83610	f	48	,, ,,	1.14	9.1
81622	m	45	,, ,,	1.15	6.5
82072	m	53	,, ,,	1.36	7.1
83417	m	43	,, ,,	1.51	9.0
82719	m	48	,, ,,	0.97	6.7
82283	m	59	,, ,,	1.12	5.1
83114	m	50	,, ,,	1.16	9.0
83584	m	53	,, ,,	0.85	8.7
81603	f	48	,, ,,	1.46	7.4
				avg 1.15	avg 7.8
82300	m	46	Prim. Op. Ca. Stomach	1.30	6.9
81764	m	64	,, ,, ,, ,,	0.81	6.5
79547	m	45	Op. Ca. Duodenum	0.87	7.4
82108	f	60	Prim. Op. Ca. Stomach	0.74	7.1
82391	f	50	,, ,, ,, ,,	0.93	6.1
81954	f	63	,, ,, ,, ,,	1.12	8.0
82018	m	51	,, ,, ,, ,,	1.28	7.2
83016	m	49	,, ,, ,, ,,	0.94	6.7
83444	m	62	,, ,, ,, ,,	1.58	10.0
83273	m	63	Inop. Ca. Stomach	1.06	15.0
81473	m	64	,, ,, ,,	1.26	4.8
80296	f	41	,, ,, ,,	1.04	10.2
75069	m	74	,, ,, ,,	0.86	7.1
25000	m	56	,, ,, ,,	0.42	4.4
80928	m	69	,, ,, ,,	1.02	6.5
80901	f	52	,, ,, ,,	0.78	5.0
				avg 1.00	avg 7.4

* Memorial Hospital Chart Number.
† AU = arginase activity which effects 50% hydrolysis under the conditions specified.

gram of tissue nitrogen.

It is apparent that there is no significant difference in arginase activity between the two groups of livers. The catalase values are quite variable in each group and although the average control value is 15% higher than that of the experimental group, the overlapping of the individual values is such as to make this difference an insignificant one.

Discussion. These negative results are of interest with respect to the work of Greenstein.[1,2,4]

The negative results are also of interest with respect to the work of Abels[5] and coworkers who demonstrated impairment of certain liver functions as an almost invariable concomitant of cancer of the gastro-intestinal tract in humans. Liver functions found to

be impaired included several which involve a variety of types of chemical reaction. Hence, it is of interest that the two functions chosen for this study should be found to be normal.

The *decrease* in arginase activity in the livers of animals bearing tumors may be limited to a few species since in no case were mice observed by Greenstein to exhibit this phenomenon. However, the decrease in catalase activities was found in both rats and mice with a wide variety of tumors but one exception. The actual magnitude of this decrease is not clear since later work by Greenstein indicates a 50% decrease in activity whereas in the first reports the decrease was 90% in many cases. In any event, it is clear that in humans the presence of adenocarcinoma of the stomach does not significantly influence the catalase content of the liver.

Conclusions. The catalase and arginase activities of liver biopsies from 16 patients

5 Abels, J. C., Rekers, P. E., Binkley, G. E., Pack, G. T., and Rhoads, C. P., *Ann. Int. Med.,* 1942, 16, 221.

has been the demonstration that tumors may influence composition or metabolism of tissues not involved anatomically.

The most striking instance of such an effect is that reported by Greenstein.[1] He showed that the catalase activity of the livers of rats and mice bearing transplanted or spontaneous tumors was markedly decreased. On removal of the tumor, the activity promptly returned to normal and a second transplant resulted in a second depression.[2] Similar although less marked effects were demonstrated on liver arginase activities in some species.[3] These results lead one to conclude that the tumor may produce systemic effects in the host. Because of the implications of such a point of view, the extension of this type of investigation to the field of human cancer may be of great importance.

This paper presents the results of catalase and arginase determinations on liver biopsies obtained from 16 patients bearing gastric cancer and 11 patients who underwent surgery for benign lesions.

Clinical Material. The patients included in this study were unselected insofar as age, sex or nutritional state were concerned. They were all admitted to the Gastric Service of the Memorial Hospital for abdominal surgery and were given the usual pre-operative care and study. Food was withheld for at least 10 hours prior to operation. The liver biopsy was taken at the start of the operation, frozen within 15 minutes, and stored in a glass, airtight container at −40°C until immediately before analysis. The biopsy was invariably a wedge-shaped piece taken from the edge of the right lobe and weighed from 300-1000 mg.[†]

Methods. The weighed, frozen tissue was dropped into 6 volumes of ice-cold 0.5 N KCl solution, containing .03 N NaHCO₃

and thoroughly ground in a glass homogenizer. One milliliter of this mixture was diluted to 100 ml with cold distilled water and used immediately for estimation of catalase activity. The remainder of the homogenized tissue was allowed to incubate for 18 hours at 5°C before measurement of arginase activity.

Catalase activity was determined by estimating the H_2O_2 decomposed by 2 ml of the fresh, dilute tissue suspension added to 5 ml of approximately 0.09 M H_2O_2 (Merck's superoxol diluted 1-100) buffered with 1 ml M/5 sodium phosphate at pH 7.4. The mixtures were shaken for 20 seconds at 25°C and the reaction stopped by the addition of 2 ml 10 N H_2SO_4. The remaining H_2O_2 was titrated with 0.04 N $KMnO_4$ and this value subtracted from a blank titration obtained when the acid was added before the tissue. Preliminary control experiments demonstrated the suitability of this test system with regard to linearity of H_2O_2 decomposed as a function of tissue concentration and of time. In no case was the availability of substrate a limiting factor.

The arginase estimations were performed exactly as described by Greenstein,[4] except that the whole tissue *brei* was used rather than the supernatant extract. Ten dilutions of each activated extract were selected so that in the half hour incubation time chosen, the most dilute suspensions hydrolyzed from 8 to 20% of the arginase whereas the more concentrated suspensions effected complete hydrolysis.

The total nitrogen in each homogenate was determined by a micro-Kjeldahl method.

Results. Table I shows the results of the activity estimations together with pertinent data on each patient. Catalase activity is expressed as milliliters of oxygen liberated from the test system per milligram of tissue nitrogen per second. One unit of arginase activity is chosen as that which will effect the splitting of 50% of the arginine present in the test system in thirty minutes at 37°C. The arginase activities are expressed in units per milli-

1 Greenstein, J. P., Jenrette, W. V., and White, J., *J. Nat. Cancer Inst.*, 1941, **2**, 282.

2 Greenstein, J. P., and Andervont, H. B., *J. Nat. Cancer Inst.*, 1942, **2**, 345.

3 Greenstein, J. P., *J. Nat. Cancer Inst.*, 1943, **3**, 419.

4 Greenstein, J. P., Jenrette, W. V., Mider, G. P., and White, J., *J. Nat. Cancer Inst.*, 1940-1941, **1**, 687.

TABLE I.
Fibrinolysis of Blood of Anterior Dog in Peptone Shock.

Protamine (mg per ½ ml blood)	Minutes after peptone injection									
	0*		2		6		18		30	
	Hr	Units[†]	Hr	Units	Hr	Units	Hr	Units	Hr	Units
.100	4	2	1	4	2	3	2	3	2‡	3
.050	24‡	0	2	3	2	3	2	3	4‡	1
.025	,,	0	2	3	2	3	2	3	4‡	1
.012	,,	0	2	3	2	3	4‡	1	∞	0
.000	,,	0	2	3	2	3	24	0.5	∞	0
Control	∞	0	2	3	2	3	24	0.5	∞	0
Total lytic activity		2		19		18		11		5

* After operation and before peptone injection.

† Arbitrary units, total lysis in 1 hr given the value 4, in 2 hr, 3, in 4 hr, 2, in 24 hr, 0.5, and partial lysis in 4 hr given the value 1.

‡ Only partial lysis (at least half the clot) in the time stated.

Fibrinogen. Armour Fraction I. *Thrombin.* Lederle's "Hemostatic Globulin."

Peptone Shock in the Anterior Dog. The dogs were anesthetized with sodium pentobarbital, arranged for recording carotid blood pressure and prepared as anterior animals according to Nolf.[8] Injections of peptone were made into one of the external jugular veins and blood samples drawn from the other into a syringe rinsed with saline but containing no anticoagulant. The fibrinolytic activity of this blood was determined by adding it to a series of protamine tubes, the same as used in the protamine-titration test for heparin. A typical determination of fibrinolytic activity is shown in Table I. The fibrinolytic activity of each blood sample is expressed as the sum of the arbitrary units of activity assigned to each tube of the protamine series according to the length of time required for the lysis of the blood in that tube. The experiment recorded here shows the typical changes in fibrinolytic activity after peptone injection, an immediate increase followed · by a slower decrease.

On the injection of peptone the blood pressure fell quite markedly in all cases but it was found that a concentration of 300 mg of peptone per kg was necessary to produce a prolonged fall in blood pressure and evidence of a prolonged activation of the serum protease.

To investigate the effect of the S.B.I. on the fibrinolysis in other experiments, part of each blood sample was added to the S.B.I. solution and pipetted to the protamine series after the control. Since the S.B.I. has some anticoagulant activity[9] a drop of thrombin was added to each tube of both control and S.B.I. series. The inhibitory effect of the S.B.I. on the fibrinolysis is well illustrated in Table II, where moderate lytic activity is completely inhibited by concentrations of S.B.I. as low as 0.34 mg per ml of blood.

In one experiment, S.B.I. (20 mg./kg) was injected into the anterior dog 2 minutes before the injection of the peptone. The injection of the S.B.I. had no effect on the blood pressure nor any effect on the fall in blood pressure after the injection of peptone. However, as shown in Fig. 1, there was a marked decrease in the degree of fibrinolysis. Also the preparation survived more than twice as long as a number of similar preparations subjected to the injection of peptone without previous treatment with S.B.I.

Since the platelets and leucocytes are believed to be involved in the production of peptone shock,[1] counts of the cellular elements were made during the experiments and are given in Table III. Platelets and leucocytes were greatly reduced after the injection of peptone, this reduction being accompanied by a marked agglutination of the platelets. The platelets and leucocytes decreased to the same level (approximately 25,000/mm³ for platelets and 2,000/mm³ for leucocytes) regardless of the dose of peptone, the initial

bearing gastric cancer did not differ significantly from those of 11 patients having benign gastric lesions. These results are in contrast to those found in animal experiments, and are in contrast to those obtained with certain other liver function tests which appear to be impaired in patients with gastric cancer.

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Activation of Serum Protease in Peptone Shock.[*]

A. EVELYN SCROGGIE,[†] L. B. JAQUES, AND M. ROCHA E SILVA.

From the Department of Physiology, University of Saskatchewan, Saskatoon, Canada, and Department of Biochemistry and Pharmacodynamics, Instituto Biologico, São Paulo, Brazil.

A mechanism for the release of histamine and heparin in anaphylactoid conditions has been previously postulated by one of us.[1] According to this theory, the anaphylotoxins are released as a direct result of the activation of a serum protease which has been described in the literature as "plasma trypsin,"[2] "lytic factor,"[3] "plasmin,"[4] "tryptase"[5] and "fibrinolysin."[6] The activation of this protease in peptone shock is indicated by an increase in the fibrinolytic activity of the blood taken from dogs in the severe stages of shock, the fibrinolysis being observed in the tubes of the protamine titration test for heparin.[7] Heparin has been shown to have an inhibitory effect on "plasma trypsin."[2] Hence the addition of protamine to the blood, as in the protamine titration test for heparin, provides conditions under which the activation of the serum protease may be demonstrated by the resultant fibrinolysis.

Fibrinolytic activity of the blood has been observed by Nolf[8] after the injection of peptone into the liverless and the anterior dog. Since the anterior dog has its inferior vena cava and thoracic aorta ligated just above the diaphragm, thus excluding the liver from the circulation, and since in the dog most of the heparin released comes from the liver, the anterior dog provides conditions *in vivo* similar to those provided *in vitro* by an excess of protamine. The anterior dog was used in our experiments to study the fibrinolytic activity of the blood in peptone shock. A second system which has been studied is the release of histamine from the leucocytes of the rabbit on the addition of peptone. The activity of the serum protease has been investigated by the use of the trypsin inhibitor isolated from unheated soya bean flour, which has been shown to be an inhibitor of the serum protease.[9]

Materials.

Soya Bean Trypsin Inhibitor (S.B.I.): We are indebted to Dr. M. Kunitz for a sample of this material recrystallized 5 times.

Peptone. Difco proteose-peptone.

Heparin (1000 units/ml) and *Protamine* (Salmine hydrochloride) were supplied by the Connaught Laboratories, University of Toronto.

1 Rocha e Silva, M., and Teixeira, R. M., PROC. SOC. EXP. BIOL. AND MED., 1946, 61, 376.

2 Rocha e Silva, M., and Andrade, S. O., *Science*, 1945, 92, 670.

3 Milstone, H., J. Immunol., 1941, 42, 109.

4 Christensen, L. R., J. Gen. Physiol., 1945, 28, 363.

5 Ferguson, J. H., *Science*, 1943, 97, 319.

6 Loomis, E. C., George, C., Jr., and Ryder, A., Arch. Biochem., in press.

7 Jaques, L. B., and Waters, E. T., J. Physiol., 1941, 99, 454.

8 Nolf, P., *Medicine*, 1938, 17, 381.

9 Tagnon, H. J., and Soulier, J. P., PROC. SOC. EXP. BIOL. AND MED., 1946, 61, 440.

TABLE III.
Platelet and Leucocyte Count of the Anterior Dog in Peptone Shock.*

No. of dog	Dose of peptone (mg/kg)	Pre-operative		Before peptone		After peptone		
		Leucocytes /mm3	Platelets × 1000/mm3	Leucocytes /mm3	Platelets × 1000/mm3	Time min.	Leucocytes /mm3 × 1000	Platelets × 1000/mm3
3	150	12,100	419	10,300	440	6	2.2	28 (72%) †
						20	5.2	70 (50)
						35	4.6	69 (51)
4	200	17,600	258	11,300	276	6	2.4	26 (68)
						19	2.9	25 (30)
						58	7.5	85 (15)
						10	5.4	38 (33)
6	300	8,400	225	4,400	229 (9%)	5	2.0	19 (73)
						37	1.2	14 (45)
8‡	300	5,100	445	5,100	426 (6%)	8	2.0	22 (39)
						26	2.4	52 (42)
	300					11	2.5	35 (7)
10	300	15,900	481	11,200	471 (5%)	4	5.4	104 (76)
						30	3.7	19 (41)
						60	4.7	68 (33)

* Leucocyte and platelet counts have been adjusted to the red cell count in the normal sample.
† Figures in parentheses refer to the percentage agglutination of the platelets.
‡ Dog injected with 20 mg S.B.I. per kg 2 minutes before injection of peptone.

combination with the inhibitor released during the process of activation.

To study the effect of protamine on fibrinolysis, a lytically active dog serum was prepared by treatment with chloroform.[10] This "chloroform serum" was used to lyse a standard fibrin clot, the lysis being inhibited by fresh dog serum which contains the natural inhibitor of the serum protease. The effect of protamine was tested on this lytic system, which, like the blood from the anterior dog in peptone shock, lacks the complicating factor, heparin.

In one experiment, a constant amount of protamine (300 μg) was incubated at 37.5°C with varying dilutions of the "inhibitor serum" for 5 minutes before the addition of the "chloroform serum" and clotting elements (0.2 ml of a 1:10 dilution of thrombin and 0.5 ml of 0.3% fibrinogen). Fig. 2 shows that for each dilution of the "inhibitor serum" the clot with protamine lysed before its control.

In another experiment, different amounts of protamine were incubated at 37.5°C with a constant "inhibitor serum" dilution for

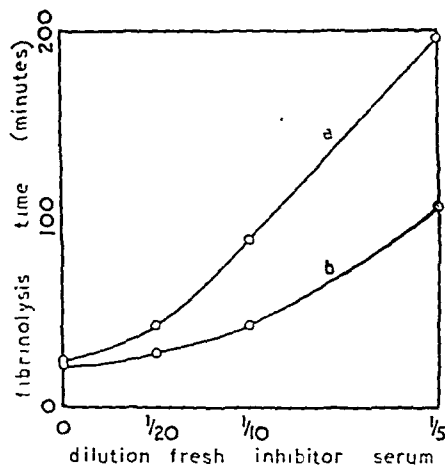10 Christensen, L. R., J. Bacteriol., 1944, 47, 471.

FIG. 2.
Effect of Protamine on Fibrinolysis. a (no protamine)—inhibitor serum + saline incubated at 37°C for 5 minutes, then 0.1 ml of chloroform serum, 0.2 ml of 1:10 thrombin and 0.5 ml of 0.3% fibrinogen added. b (+ protamine)—inhibitor serum + protamine incubated at 37°C for 5 minutes before the addition of chloroform serum + thrombin + fibrinogen.

5 minutes before the addition of the "chloroform serum" and clotting elements. Table IV A shows that increasing the concentration of the protamine in the clot increased the
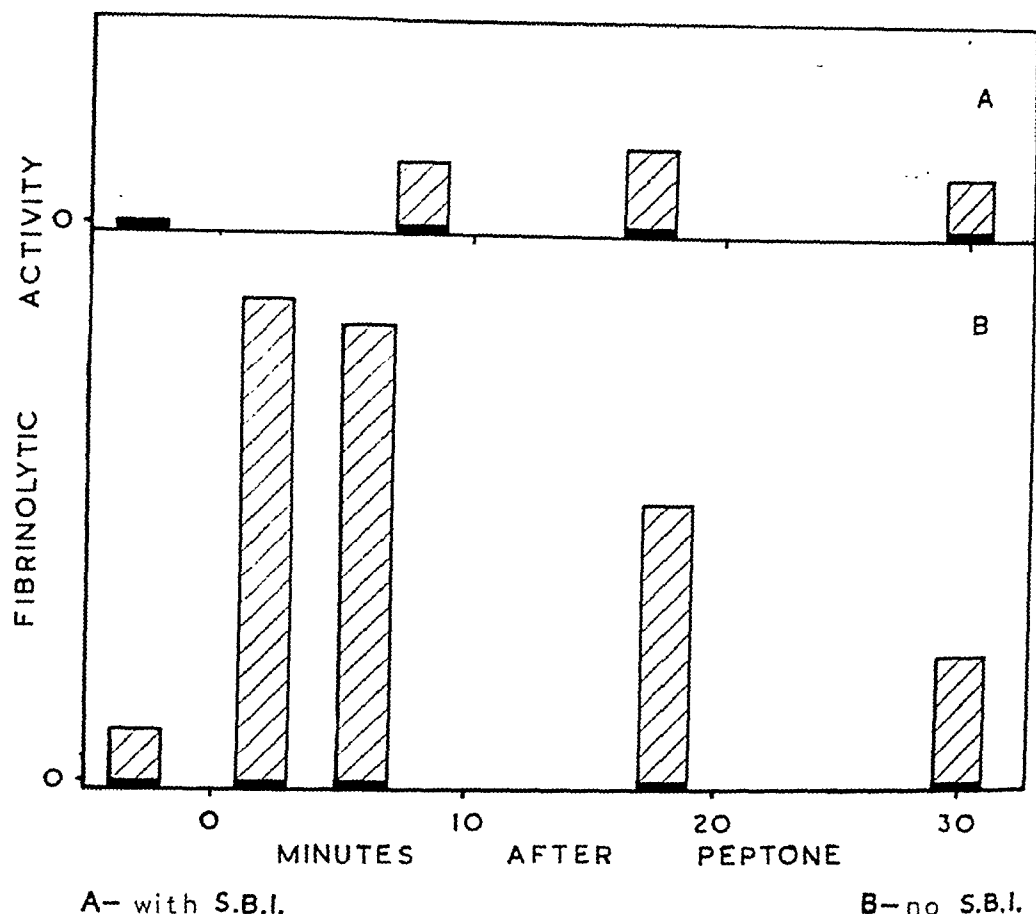
A— with S.B.I.   B— no S.B.I.

FIG. 1.

Inhibitory Effect of S.B.I. *in vivo* on Fibrinolysis. A—Anterior dog injected with S.B.I. (20 mg/kg) 2 minutes before the injection of peptone (300 mg/kg). B—Anterior dog injected with peptone (300 mg/kg).

level of the cells, the previous injection of the S.B.I., or the extent of the fibrinolysis produced.

The Effect of Protamine on Fibrinolysis. In peptone shock in the intact dog, the addition of protamine to the blood enables fibrinolysis to be observed. This could be due to the neutralizing effect of protamine on heparin, since heparin has been shown to antagonize the serum protease.[2] However, the fact that fibrinolysis occurred first in the tube with the highest concentration of protamine, regardless of the quantity of heparin present, indicated that protamine might have some other action promoting fibrinolysis as well as that of neutralizing

TABLE II.

Effect of S.B.I. on Fibrinolysis in Blood of Anterior Dog after Peptone.

S.B.I. (mg/cc blood)	Lytic activity of control blood	Lytic activity with S.B.I.
1.7	24	0
0.83	16	0
0.67	14	0
0.34	14	0
0.17	14	2

heparin. The proteolytic activity of the blood is thought to be inhibited normally by a proteolytic inhibitor which is present in serum, so that activation of the protease occurs when the inhibitor is removed. Protamine might act by competing with the free enzyme for

a plateau in about 20 minutes. When the blood had been incubated with S.B.I. previously, the addition of peptone was not followed by a rapid increase in plasma histamine. However, the S.B.I. did not completely inhibit the release of histamine, which proceeded at a slow rate.

Discussion. As shown above, we have found that S.B.I. inhibits fibrinolysis due to the serum proteolytic enzyme activated by chloroform, while protamine inhibits the natural serum inhibitor of this enzyme. Since S.B.I. inhibited the lysis occurring in peptone shock in the anterior dog, while the addition of protamine increased the lysis, it is evident that the fibrinolysis due to peptone is due to the activation of the plasma enzyme. S.B.I. inhibited the release of histamine by peptone from rabbit blood cells. This suggests that activation of serum protease is a step in the release of histamine by rabbit blood cells. Unfortunately sufficient S.B.I. was not available for the corresponding experiment in the dog to test the effect on liberation of histamine by body cells.

The action of the strongly basic protamine on fibrinolysis of both blood and fibrin clots lends support to the hypothesis of Ferguson[5] and others that the inhibitor of the plasma proteolytic enzyme is a polypeptide with acidic groups analogous to those of heparin or that heparin is a prosthetic group for this inhibitor.

The strikingly uniform reactions of platelets and leucocytes, as far as their counts are concerned, despite wide variation in the fibrinolytic effect produced, would indicate that these cell elements are neither concerned with the production of, nor affected by, the activation of the serum protease. However, there is some evidence that this may not be the case. Smears from the cut edge of fragments of lung tissue, prepared according to the technique described by Rocha e Silva[13] showed that when fibrinolysis was marked, disintegration of the platelet clumps in the lung vessels had proceeded to a degree where only dark-staining granules remained as remnants of the disintegrated platelets. When fibrinolysis was weak or absent, the disintegration of the platelets in the clumps was very slight or absent. Thus it appears that although the counts of circulating platelets and leucocytes may not indicate any relationship to fibrinolysis, the smear technique for examining clumps of platelets and leucocytes may indicate the true relationship of these cell elements to fibrinolysis and other results of the injection of peptone.

Summary. The relationship of the serum protease to the action of peptone was investigated using soybean trypsin inhibitor and protamine. Soybean trypsin inhibitor inhibited fibrinolysis by chloroform serum (activated serum protease). It inhibited the fibrinolysis occurring in peptone shock in the anterior dog and also the release of histamine from rabbit blood cells *in vitro* on the addition of peptone. Protamine accelerated fibrinolysis in the anterior animal and also lysis by the serum protease in the presence of the natural serum protease inhibitor.

13 Rocha e Silva, M., Porto, A., and Andrade, S. O., *Arch. Surg.*, 1946, 53, 199.

TABLE IV.

Effect of Protamine and S.B.I. on Fibrinolysis.*

A			B	
Protamine (μg)	Inhibitor serum (1/5 dilution) (ml)	Lysis time (min)	S.B.I. (mg)	Lysis time
—	—	27	—	6.5 min
30	—	27	3.0	(no lysis in 12 hr)
—	.1	216	2.0	,, ,, ,, ,, ,,
300	.1	138	1.0	,, ,, ,, ,, ,,
30	.1	206	0.1	,, ,, ,, ,, ,,
3	.1	2166	0.01	(lysis within 12 hr)
			0.001	,, ,, ,, ,,

* Standard fibrin clot containing 0.2 ml of a 1:10 dilution of hemostatic globulin and 0.5 ml of 0.3% fibrinogen (Armour Fraction I).

A—Total volume 1.5 ml containing 0.4 ml chloroform serum.

B—Total volume 1.4 ml containing 0.1 ml chloroform serum.

speed of lysis. S.B.I. added to a lytically active clot, inhibited the lysis, as shown in Table IVB. Tagnon[9] has reported similar observations.

Effect of S.B.I. on release of histamine from rabbit blood by peptone. Blood was taken into heparin from rabbits by cardiac puncture. Part of the blood was incubated for 5 minutes at room temperature with S.B.I. (0.5 mg per ml of blood) while 2 controls were incubated with saline. To the blood + S.B.I. and to one of the controls, peptone was added (15 mg per ml of blood) while to the other control saline was added. The peptone used had been freed of histamine by treatment with permutit.[11] The mixtures were incubated for various times and histamine extractions performed on their plasmas by the method of Code.[12] The assay on the guinea pig ileum showed that in a number of experiments with an incubation time of 15 minutes, there was an inhibition by S.B.I. of the release of histamine by peptone. Little inhibition was demonstrated by the S.B.I. with an incubation time of 30 minutes.

The effect of time of incubation on the release of histamine is shown in Fig. 3. Three mixtures, a and c containing blood + saline and b containing blood + S.B.I., were incubated for 5 minutes at room temperature. Peptone was added to a and b and saline to c, the concentrations of S.B.I. and peptone

11 Gotzl, F. R., and Dragstedt, C. A., J. Pharm. and Exp. Ther., 1941, 74, 33.
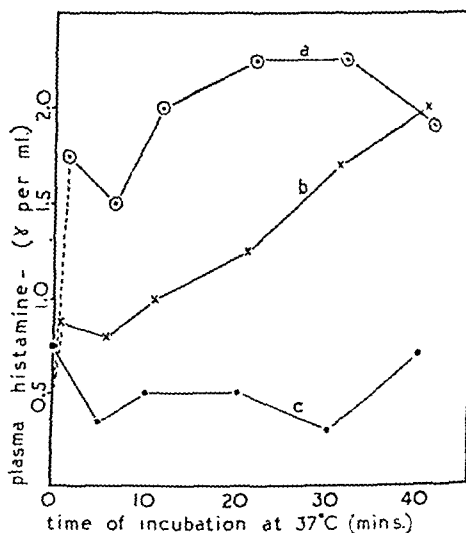
12 Code, C. F., J. Physiol., 1937, 89, 257.

FIG. 3.

Effect of S.B.I. on the Release of Histamine from Rabbits' Blood by Peptone. a—Blood + Peptone. b—Blood + S.B.I. + Peptone. c—Blood + Saline.

in the blood being the same as above. From these mixtures incubated at 37°C, samples were taken at various times and packed in snow until all the samples were taken. These were centrifuged simultaneously and their plasmas extracted for histamine. With saline alone added to the blood, plasma histamine remained constant, the variation shown in the samples indicating the degree of accuracy of the method used (±0.2 μg histamine). With peptone alone added to the blood, the release of histamine initially was rapid, slowing to

TABLE I.
Distribution of St. Louis and Japanese Encephalitis Viruses in the Hamster After Feeding.

Tissue tested	St. Louis virus	Japanese virus
(3rd to 8th day)		
Buccal mucosa	3/10	2/10
Tongue	7/10	6/10
Larynx	5/10	5/10
Cervical lymph nodes	8/10	7/10
Esophagus	3/10	3/10
Stomach	0/10	0/10
Small gut	7/10	1/10
Lymphoid follicles of small gut	6/10	4/10
Large gut	6/10	3/10
Mesenteric lymph node	8/10	4/10
Inguinal lymph node	5/10	5/10
Nasal mucosa	4/10	8/10
Olfactory bulbs	3/10	8/10
Cerebrum	5/10	7/10
Brain stem	4/10	6/10
Spinal cord	4/10	6/10
Spleen	6/10	
Striated muscle	0/10	
(1st to 5th day)		
Blood	10/46	30/38

Denominator indicates number of tests made; numerator indicates number positive.

ster after feeding, being recoverable from many tissues, including the blood, a fact which obscures both the site or sites of multiplication of virus, and the sequence of events in the pathogenesis of the infection.

Viremia has been present as early as the second day after feeding with St. Louis virus, and the first day in the case of Japanese, but it is intermittent in the former case and with neither virus has it been demonstrable after the fifth day. The presence of virus in the blood and certain other tissues before it is recoverable in the central nervous system (3rd to 8th day) suggests that multiplication may occur outside the central nervous system.

Both of these viruses have been recovered by swabbing the mouths of hamsters on the sixth to ninth days after feeding, when clinical signs were present. The cotton swabs were washed out in serum-broth, penicillin was added (2000 units per cc) and the supernate after centrifugation was injected intracerebrally into mice. By this technic St. Louis virus was recovered in 3 of 5 attempts in 3 hamsters, and Japanese virus in 12 of 13 tests on 12 hamsters. The latter virus has also been found in the mouth 7 times in 6 hamsters on the first to fifth day after feeding, before any clinical signs of infection were apparent. St. Louis virus was isolated from the mouths of hamsters (2 of 3 on third day; one of 2 on fourth day) that had been injected intracerebrally.

We are unaware of any direct evidence at present that transfer of virus to or from the alimentary canal is significant in the epidemiology of these encephalitides. It is of interest to note, however, that evidence has been submitted[6] for the presence of St. Louis virus in the nasopharyngeal washings of patients and has been recovered from nasal washings of an intracerebrally inoculated horse.[7] Venezuelan equine encephalomyelitis virus has been recovered from human nose and throat washings.[8,9,†] It appears that the possibility of natural hosts of these viruses acquiring infection through the alimentary tract, and transmitting either by droplets from the mouth or by the feces, deserves careful consideration.

6 Sulkin, S. E., Harford, C. G., and Bronfenbrenner, J., PROC. SOC. EXP. BIOL. AND MED., 1939, 41, 327.

7 Cox, H. R., Phillip, C. B., and Kilpatrick, J. W., Pub. Health Rep., U. S. P. H. S., 1941, 56, 1391.

8 Casals, J., Curnen, E. C., and Thomas, L., J. Exp. Med., 1943, 77, 521.

9 Lennette, E. H., and Koprowski, H., J. A. M. A., 1943, 123, 1088.

† There are also several pertinent references in the Japanese literature, the originals of which we have been unable to obtain.

16082 P

Susceptibility of the Hamster to St. Louis and Japanese Encephalitis Viruses by Feeding.*

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It was reported previously[1] that hamsters, when allowed to feed upon St. Louis encephalitis virus, either die with encephalitis, or develop an immunity as evidenced by appearance of serum antibody and resistance to intracerebral inoculation. Subsequent tests have indicated that hamsters, fed Japanese encephalitis virus react in much the same way. The present report describes the extent of the infection in hamsters following feeding with St. Louis (Hubbard strain) and with Japanese (Nakayama strain) encephalitis viruses, as determined by subinoculation of mice with various tissues.

The possibility of infection by way of the alimentary tract with neurotropic viruses, other than those of poliomyelitis and encephalomyelitis of mice, has received some attention. Infection of mice by introduction of St. Louis virus into the alimentary tract has been described by 2 groups of investigators,[2,3] and both report development of immunity in survivors. The latter group also fed 2 hamsters with St. Louis virus and found neutralizing antibody some days later.[4]

In our experiments hamsters were induced to eat virus by starving them overnight, and offering them the heads of infected mice. Tissues to be tested for virus were carefully removed from exsanguinated animals with separate sets of instruments, emulsified in a minimum quantity of broth to which 10% normal sheep serum had been added, centrifuged, and the supernatant inoculated into 3 or 4 mice each. In the case of bacterially contaminated tissues such as the intestine, the specimens were washed repeatedly in sterile physiological saline and were thereby rendered sufficiently free of bacteria to allow intracerebral inoculation.

In most of the experiments a second intracerebral passage to mice was made with the brain tissue of any mouse found ill or dead, in which there was doubt concerning the cause. A simple technic, as described elsewhere,[5] was used. Blood specimens were taken by cardiac puncture and immediately injected intracerebrally into mice.

Preliminary tests for St. Louis virus in hamster tissues included the large and small gut, the lymphoid follicles present in the wall of the latter, and the mesenteric lymph node. Virus was found on occasion in one or more of these tissues from the first to the ninth day after the hamster had fed upon virus but the incidence was greatest in the 2 lymphoid tissues on the fourth or fifth day. Three different strains of virus recovered in these experiments from intestine or associated lymphoid tissue of hamsters were identified as St. Louis virus in neutralization tests with immune rabbit serum.

Ten attempts to recover virus from intestinal contents of hamsters fed upon St. Louis virus included both filtered and unfiltered samples, but were unsuccessful.

The results of more extensive experiments are presented in Table I. It is evident that both viruses become widespread in the ham-

* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

1 Gordon, F. B., *J. Bact.*, 1944, **47**, 465 (abstract).

2 Harford, C. G., Sulkin, S. E., and Bronfenbrenner, J., Proc. Soc. Exp. Biol. and Med., 1939, **41**, 332; Harford, C. G., and Bronfenbrenner, J., *J. Inf. Dis.*, 1942, **70**, 62.

3 Mezera, R. A., Broun, G. O., Muether, R. O., and LeGier, M., Proc. Soc. Exp. Biol. and Med., 1941, **48**, 357.

4 Broun, G. O., LeGier, M., Mezera, R. A., and Muether, R. O., Proc. Soc. Exp. Biol. and Med., 1941, **48**, 310.

5 Schabel, F. M., Jr., and Gordon, F. B., *Science*, 1947, **106**, 549.

TABLE I.
Mating Experiments on Rats on a Purified Diet Containing About 100-400 Micrograms Tocopherols per 100 g, According to Age and Tocopherol Supplement.

Alpha tocopherol acetate supplement	No. of experiments	No. of pregnancies	% Implantations	No. of Litters	No. of Resorptions	Litters in % of pregnancies
Age 53-150 Days.						
None	47	31	66	3	28	10
Single dose of .5-1 mg	48	36	75	9	27	25
,, ,, ,, 1.1-2 ,,	51	40	78	23	17	58
,, ,, ,, 2.1-3 ,,	11	9	82	8	1	89
4 mg per 100 g diet	55	48	87	48	0	100
Age 151-240 Days.						
None	53	29	56	4	25	14
Single dose of .5-1 mg	37	27	73	9	18	33
,, ,, ,, 1.1-2 ,,	47	30	64	20	10	67
,, ,, ,, 2.1-4 ,,	18	15	83	14	1	93
4 mg per 100 g diet	22	16	73	15	1	93
Age 241-360 Days						
None	22	4	18	2	2	?
Single dose of .5- 2 mg	24	16	66	4	12	25
,, ,, ,, 2.1- 4 ,,	14	7	50	4	3	57
,, ,, ,, 4.1-10 ,,	26	15	58	8	7	53
4 mg per 100 g diet	32	24	75	24	0	100
Age 361 Days and Over.						
Single dose of 0 - 3 mg	20	2	10	0	2	0
,, ,, ,, 3.1-10 ,,	29	12	41	2	10	18
"Massive" doses	50	11	22	9	2	82
4 mg per 100 g diet	33	20	61	17	3	85

was followed by weight gain and thereafter by gradual weight loss, a resorption gestation was recorded. In 22 not entirely clear cut cases, autopsies were performed. A litter was registered regardless of the number of dead and living young. Animals which persistently failed to become pregnant were excluded from the charts in accordance with the practice of Evans and Burr.[7] Only females of 150 g or over were used for breeding.

With this technique, pregnancy was observed in 36 out of 43 mating tests (84%) in rats on "Evans-Burr" diet, and in 87% of the younger rats on the complete diet. These data are in good agreement with Evans' and Burr's[7] observation on the implantation rate in a rat colony kept on a complete diet, and with those reported by Goettsch and Pappenheimer.[8]

In the earlier experiments, males of proven fertility kept on Rockland rat diet were used. In over 900 of the experiments, however, the males raised on the complete simplified diet were mated. In case no pregnancy was ob-

served, the result was charted only if the male was fertile before and after the negative test.

The females were used repeatedly. On the 5th day after the mating had commenced and after the male had been removed, they were either left on the diet or they were given single supplements of alpha tocopherol acetate diluted with 30 g of the experimental diet. The cages were cleaned before the supplements were offered, and no additional food was given until the supplement was completely consumed, usually within 3 days. The single doses amounted to .5-3 mg alpha tocopherol during the first 5 months of life, to .5-4 mg from the fifth to the eighth months, and thereafter to .5-10 mg.

Fifteen female rats kept on the deficient ration, were given 1-2 drops (30-60 mg) of alpha tocopherol acetate at intervals of 1-2 weeks, after they had reached the age of one year.

After a resorption gestation or after the weaning of a litter (towards the end of the fourth week), at least 4 weeks elapsed before the female was mated again.

It is probable that some pseudopregnancies were recorded as resorptions, because the

[7] Evans and Burr, The Antisterility Vitamine Fat Soluble E, Univ. Cal. Press, Berkeley, 1927.

[8] Goettsch and Pappenheimer, J. Nutrition, 1941, 22, 463.

16083

Influence of Alpha Tocopherol on Implantation in Old Rats.*

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Investigations concerned with the fertility of female rats on vitamin E deficient diets have been mainly focused on the "resorption gestation." The question of implantation in vitamin E deficiency has received much less attention.[1]

In these experiments, the rate of implantation of female rats at different age periods on a vitamin E deficient diet was compared with that of rats on the same diet completed by addition of alpha tocopherol.

Methods. 1130 mating experiments were carried out on 293 albino rats of a highly inbred colony. The ancestry of these rats had been kept for 7 or 8 generations on a modified "Evans-Burr" diet containing 20% commercial lard. When these females gave birth to a litter, they were immediately placed on a simplified diet on which they, their offspring and subsequent generations were kept without interruption.

The diet consisted of:

	parts
commercial lard	10
crude casein	30
cerelose	54
celluration	2
salt mixture	4
	mg/kilo
pyridoxine	4
thiamine chloride	2
riboflavin	4
choline	1000
vitamin K	4
p-aminobenzoic acid	300
calcium pantothenate[†]	10
oleum percomorphum	200

Its tocopherol content was repeatedly checked according to the method of Kaunitz-Beaver[2] and was found to be below .4 mg per 100 g of diet. From these determinations and from those of Quaife and Harris,[3] it can be estimated that the daily intake of the adult rats was in the neighborhood of 20-40 micrograms of tocopherols, because lard was the only measurable source of tocopherol in the diet. The latter was freshly prepared at least once weekly and kept under refrigeration. Five generations of rats raised on this diet were used for the tests. The diet had previously proved to be satisfactory in studies of growth and bioassays (Kaunitz;[4] Kaunitz and Beaver[5]).

Controls derived from the same colony were kept on the same diet augmented by 4 mg synthetic dl alpha tocopherol acetate[‡] per 100 g diet. The weights of these animals, charted according to Zucker and Zucker,[6] were satisfactory. Females of the first through fifth generation were used approximately in the same proportion as those on the deficient diet.

Mating was carried out by leaving the female for 5 days with the selected male. On and subsequent to the 15th day after mating had begun, the females were examined for the "implantation sign" and their weight was taken on the same scale daily except for occasional omissions. If the implantation sign

tocopherol acetate.

2. Failure of implantation was observed after the eighth month in females without tocopherol supplements. With continuous tocopherol administration, the implantation rate was normal after one year. Single postmating tocopherol supplements increased the implantation rate in older rats significantly. The requirements necessary for successful implantation in older rats vary widely and increase steeply with age.

3. Infections of the uterus and the tubes were frequently observed in the deficient rats, but not in those on the complete diet. Presence of infection does not explain the higher implantation rate after single postmating tocopherol doses.

4. The changes leading to the failure of implantation are prevented, but once present, only partly counteracted by alpha tocopherol acetate.

16084 P

Amputation of the Canine Atrial Appendages.*

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Thrombus formation in the left atrial appendage and embolization therefrom are frequent sequelae of rheumatic mitral stenosis. Surgical excision of the thrombus-containing appendage suggests itself as a possible therapeutic approach to this problem. As a preliminary step experiments were performed to determine the effect of amputation of one or both atrial appendages in normal dogs.

Methods. The surgical procedures under anaesthesia, consisted essentially of exposure of the heart through an incision in the left fifth or sixth intercostal space, pericardiotomy, presentation of the atrial appendage with an Allis clamp, isolation of the appendage from the atrium with a right angle clamp or curved hemostat, ligation of the base of the appendage proximal to the clamp and finally amputation of the appendage. Excision of the left atrial appendage was accomplished with the animal in the left lateral position.

Electrocardiograms were obtained pre-operatively and at varying intervals during the post-operative period. The animals were sacrificed and necropsy performed 4 to 12 weeks following surgery. Eight animals were used, 5 undergoing left, 2 right and one bilateral atrial appendectomy.

Results. Seven of the 8 dogs survived the operation. The first animal developed respiratory difficulty and died 15 minutes after closure of the chest. All the surviving animals made uneventful recoveries. Serial electrocardiograms failed to reveal abnormal atrial rhythms or evidence of atrial injury except in one dog which developed transient intra-atrial block. Necropsy revealed complete endothelialization at the site of the atrial scar. No subjacent mural thrombi were found. Pericardial and pleuro-pericardial adhesions occurred.

Conclusion. Excision of one or both atrial appendages is feasible in dogs.

nature of these experiments excluded verification by autopsy in the majority of the cases. A further source of error was the repeated administration of tocopherol to the same rat. Both these sources of error should reduce the number of rats in which failure of implantation was recorded. The statistical differences between the groups thus became even more significant.

Experiments. In Table I are computed the results of the mating tests in relation to age and tocopherol supplement. After the 240th day there is an abrupt reduction in the percentage of implanting in the animals without tocopherol supplements which is in agreement with Emerson's and Evans' observations. Single supplements of .5-2 mg after the eighth, but before the twelfth months, and doses of 3-10 mg after one year increased the rate of implantation significantly. The "mean fertility dose" of the animals of 8-12 months was roughly 3-4 mg, and after one year litters were observed only twice with single doses up to 10 mg.

When "massive doses" of tocopherol (30-60 mg in intervals of 1-2 weeks in addition to the "complete" diet) were administered to rats after at least one year on the deficient diet, about one-half of the animals gave birth to litters, but only after repeated matings. The implantation rate of the females that had received the diet plus alpha tocopherol throughout life was still practically normal at that time.

One group of 27 rats had been kept on Rockland rat diet during the first 2 months of life and thereafter on the experimental diet. At the age of 12 months, no implantations could be observed after repeated matings. When now a single dose of 6 mg alpha tocopherol acetate was offered after a single mating, litters were observed in about 2/3 of the experiments.

Discussion. Evans and Burr[7] have discussed possible pitfalls in the evaluation of implantation data obtained from rats on vitamin E deficient diets. Failures may be caused by toxic substances in the diet or by deficiencies other than vitamin E. These sources of error can hardly explain the de-crease in the implantation rate in these experiments, because appropriate single post-mating supplements of tocopherol increased the percentage of pregnancies significantly, and breeding was normal when the diet was completed by alpha tocopherol.

Infections of the uterus and the tubes were frequently observed by us in the older rats on the deficient diet. Similar observations have previously been reported by Emerson and Evans.[1] In some instances, the uterus contained 5-10 cc thick purulent material. Purulent fistulas in the inguinal region, originating from uterine infections, were observed 4 times. No purulent infections were noted in the rats on the complete diet. While infection of the genital organs may have prevented pregnancies in some of the rats, its occurrence can be excluded in those rats which responded to single post-mating doses of alpha tocopherol. Infection of the uterus and the tubes would have interfered with both the fertilization and the implantation of the ovum.

The failure of implantation in the rats of over 8 months of age indicates that, in this age group, tocopherol is essential for the implantation of the previously fertilized ovum. The requirements appear to increase with age and there are great individual variations.

Animals that had been administered constant tocopherol supplements, had a practically normal implantation rate after one year of age; among the group receiving "massive doses" after one year or more on the deficient diet, about one-half of the rats gave birth to litters, but only when mating was carried out repeatedly; the same age group on the basic diet with single tocopherol supplements of 3-10 mg had frequent resorption gestations but only 2 litters. This indicates that the changes leading to failure of implantation are prevented but, once present, only partly cured by alpha tocopherol.

Summary. 1. The implantation rate in female rats on a purified diet containing about .1-.4 mg of tocopherols per 100 g diet was compared with the implantation rates in animals on the same diet receiving single and continuous supplements of synthetic dl alpha

tocopherol acetate.

2. Failure of implantation was observed after the eighth month in females without tocopherol supplements. With continuous tocopherol administration, the implantation rate was normal after one year. Single post-mating tocopherol supplements increased the implantation rate in older rats significantly. The requirements necessary for successful implantation in older rats vary widely and increase steeply with age.

3. Infections of the uterus and the tubes were frequently observed in the deficient rats, but not in those on the complete diet. Presence of infection does not explain the higher implantation rate after single post-mating tocopherol doses.

4. The changes leading to the failure of implantation are prevented, but once present, only partly counteracted by alpha tocopherol acetate.

16084 P

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Thrombus formation in the left atrial appendage and embolization therefrom are frequent sequelae of rheumatic mitral stenosis. Surgical excision of the thrombus-containing appendage suggests itself as a possible therapeutic approach to this problem. As a preliminary step experiments were performed to determine the effect of amputation of one or both atrial appendages in normal dogs.

Methods. The surgical procedures under anaesthesia, consisted essentially of exposure of the heart through an incision in the left fifth or sixth intercostal space, pericardiotomy, presentation of the atrial appendage with an Allis clamp, isolation of the appendage from the atrium with a right angle clamp or curved hemostat, ligation of the base of the appendage proximal to the clamp and finally amputation of the appendage. Excision of the left atrial appendage was accomplished with the animal in the left lateral position.

Electrocardiograms were obtained pre-operatively and at varying intervals during the post-operative period. The animals were sacrificed and necropsy performed 4 to 12 weeks following surgery. Eight animals were used, 5 undergoing left, 2 right and one bilateral atrial appendectomy.

Results. Seven of the 8 dogs survived the operation. The first animal developed respiratory difficulty and died 15 minutes after closure of the chest. All the surviving animals made uneventful recoveries. Serial electrocardiograms failed to reveal abnormal atrial rhythms or evidence of atrial injury except in one dog which developed transient intra-atrial block. Necropsy revealed complete endothelialization at the site of the atrial scar. No subjacent mural thrombi were found. Pericardial and pleuro-pericardial adhesions occurred.

Conclusion. Excision of one or both atrial appendages is feasible in dogs.

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Perirenal Hemorrhage in Ureter Ligated Dogs Receiving Phthalylsulfathiazole.

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Reports of toxic manifestations following the administration of succinylsulfathiazole or phthalylsulfathiazole are infrequent and are considered to be of little consequence. In the course of some experiments on the treatment of experimental uremia[1] it was noted that the combination of ureter ligation in dogs and the oral administration of phthalylsulfathiazole resulted in hemorrhage beneath the fascial sheath covering the perirenal fat. Further studies were done in an attempt to discover the cause of this bleeding. The results of these studies are reported here.

Procedure. The animals used were mongrel dogs weighing 6 to 14 kg. Six test animals received 0.5 to 0.75 g/kg/day of phthalylsulfathiazole in oral divided doses for 3-6 days before the ureters were ligated and thereafter until the death of the animal. In addition, the ureters of 6 control dogs were ligated; the operation in both cases was done under ether anesthesia with morphine-atropine premedication, except Dogs 6, 7, and 9 which were anesthetized with pentobarbital sodium (30 mg/kg). The ureters were located and doubly ligated with silk about one cm from their entrance into the bladder. The animals were observed until their death.

In 4 of the test animals, (No. 8, 10, 11 and 12) blood sulfathiazole levels* were determined within 24 hours prior to death according to the method of Bratton and Marshall.[2] Serial blood phenol determinations, according

to the method of Bernhart and Schneider,[3] were done in some of the test and control animals to follow the course of the uremia. In 3 dogs receiving the phthalylsulfathiazole (No. 10, 11 and 12) daily, pre- and postoperative prothrombin times according to the method of Quick,[4] red blood cell and platelet counts, and bleeding, clotting, and clot retraction times were done. Autopsies were done on all animals as soon after death as possible.

Results. All animals in which there was evidence that urine flow had been reestablished were omitted from the series. The significant pathologic findings are recorded in Table I. Of the 6 ureter ligated animals receiving phthalylsulfathiazole, 4 had gross hemorrhage beneath the perirenal sheath (Fig. 1), and of these, 3 had free blood in the abdomen. Of the remaining two test animals, one had a marked increase in vascularity of the perirenal sheath with a small amount of perirenal hemorrhage, and the other animal had a moderate increase in perirenal vascularity without perirenal hemorrhage.

Four of the control animals, without sulfonamide administration, showed moderately increased perirenal vascularity at autopsy, but none showed perirenal sheath hemorrhage. One had a small amount of old hemolyzed blood in the abdomen, but since this could not be attributed to a perirenal lesion it was thought to be due to the operative procedure.

Of the 3 test animals in which hematologic studies were done, one showed perirenal sheath hemorrhage and free blood in the abdomen, one showed a marked increase in perirenal vascularity without hemorrhage, and one showed a moderately increased perirenal vascularity. None of these animals showed any

1 Wallace, S. L., Little, J. M., and Bobb, J. R. R., in preparation.

* We are indebted to the staff of the Clinical Chemistry Laboratory for the sulfathiazole determinations reported in this paper.

2 Bratton, A. C., and Marshall, E. K., Jr., *J. Biol. Chem.,* 1939, **128,** 537.

3 Bernhart, F. W., and Schneider, R. W., *Am. J. Med. Sci.,* 1943, **205,** 636.

4 Kracke, R. R., *Diseases of the Blood,* 2nd Ed., J. B. Lippincott Co., Philadelphia. 1941.

TABLE I

Dog No.	Increased vascularity of perirenal sheath	Hemorrhage beneath perirenal sheath	Free blood in abdomen	Capsular hemorrhage	Kidney substance hemorrhage
		Pathologic findings			
		Control			
1	Moderate	None	None	None	None
2	"	"	"	"	"
3	"	"	"	"	"
4	None	"	Yes*	"	"
5	Moderate	"	None	"	"
6	None	"	"	"	"
		Test			
7	**	Yes	None	None	None
8	**	"	Yes	"	"
9	**	"	"	"	"
10	**	"	"	"	"
11	Marked	Small amt	None	"	"
12	Moderate	None	"	Punctate	"

* This animal had 50-75 cc of old, hemolyzed blood in its abdomen with no observable origin. There were no changes in the perirenal tissues.

** Because of the perirenal sheath hemorrhage in these animals, it was not possible to determine if they had increased vascularity of the sheath.

hematologic abnormalities either pre- or post-operatively. In addition, none of the test animals during life or at autopsy showed any evidence of hemorrhage in any other tissue that was not also seen in the control animals. All animals showed punctate subendocardial hemorrhage on the valves and ventricular wall.

The average post-operative survival time of the animals receiving phthalylsulfathiazole was 79 hours with a range of from 54 to 118 hours. The average post-operative survival time of the control animals was 72 hours, with a range of from 37 to 109 hours. The post-operative course in the two groups was essentially the same, except that some of the animals subsequently showing free blood in the abdomen died more acutely, presumably of shock.

There was no significant difference in the blood phenol levels before death in the two series of animals. The blood sulfathiazole levels taken before death averaged 2.9 mg %, with a range of 2.5 to 3.4 mg %. The combined form of sulfathiazole averaged 0.1 mg %.

Discussion. The principal difference between the control and test groups of animals was the presence of hemorrhage beneath the perirenal sheath and free blood in the peritoneal cavity in the test group. We have found in other experiments (unpublished) that such hemorrhage does not occur with phthalylsulfathiazole administration to normal dogs, to dogs subjected to bilateral nephrectomy, or to dogs with ureter ligation in which the urine flow is reestablished. Therefore the responsible factors involved in producing the hemorrhage are the administration of phthalylsulfathiazole and the presence of ureteral obstruction with intact kidneys. Neither factor alone is sufficient.

The hemorrhage could be due either to hematologic changes or to vascular changes or to a combination of the two. The complete absence of abnormal hematologic findings suggests that vascular abnormalities are responsible. It appears that the increased perirenal vascularity accompanying ureteral ligation is a predisposing factor for hemorrhage upon phthalylsulfathiazole administration, but the nature of the highly localized vascular changes is unknown.

Winternitz and Katzenstein,[5] in a review of the experimental pathology of ureteral obstruction, report that at autopsy the kidney capsular vessels were prominent and congested and that the surface of the kidney in many

5 Winternitz, M. C., and Katzenstein, R., *Yale J. Biol. and Med.*, 1940, **13**, 15.
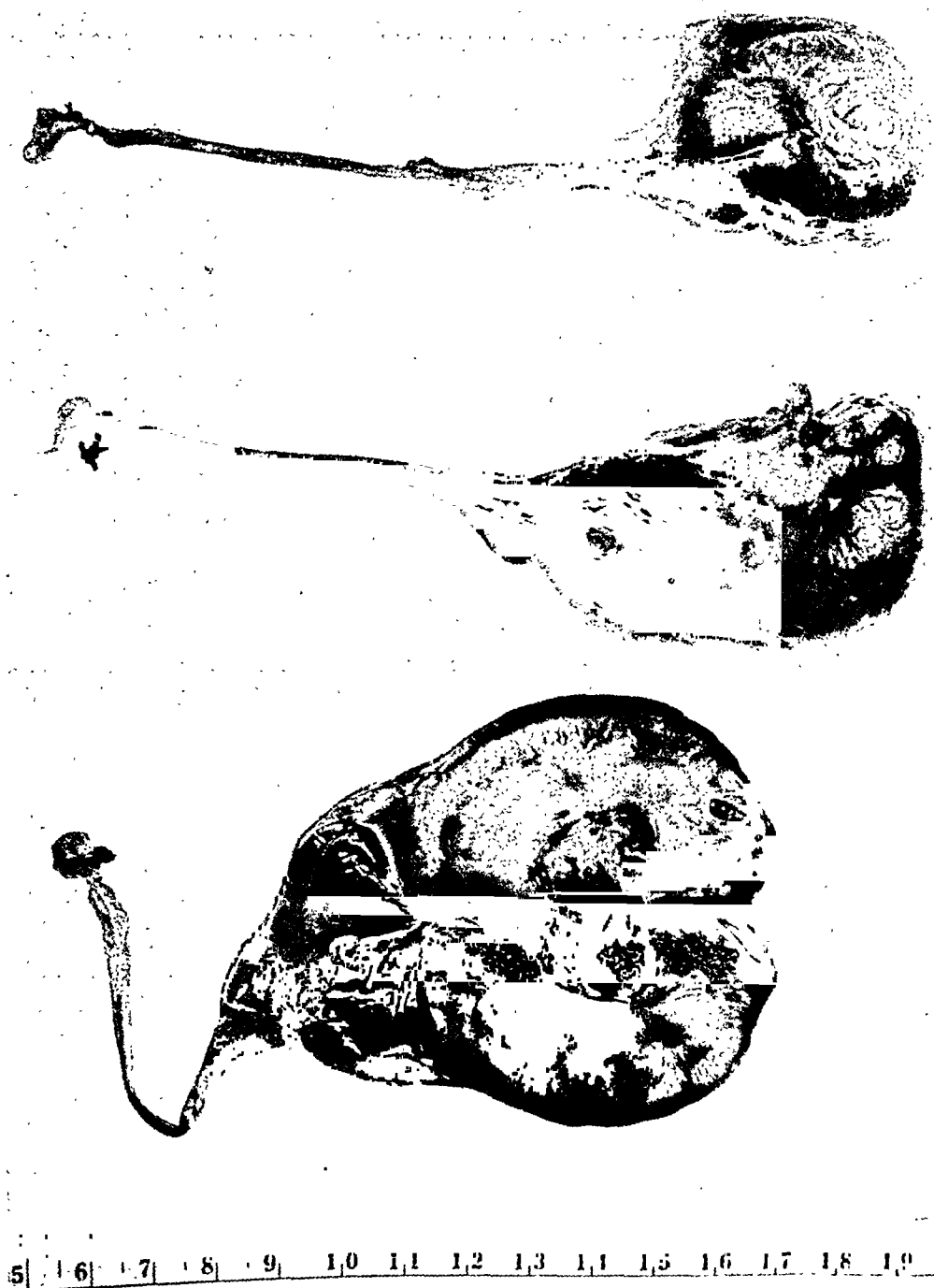
Fig. 1.

Hemorrhage beneath the fascial sheath covering the perirenal fat in ureter ligated dogs which received phthalylsulfathiazole.

cases was covered with a dark red friable exudate which usually extended into the surrounding perirenal adipose tissue. In none of their animals was perirenal hemorrhage reported. There was also no mention of such hemorrhage after ureteral ligation in the report of Harrison and Mason.[6]

Mattis. Benson and Koelle[7] reported that in one experiment they gave 1.0 g/kg of phthalylsulfathiazole intraperitoneally daily for 6 days to a monkey. The animal died on the sixth day with severe abdominal pain, hematuria. crystalluria, nausea, vomiting, weakness. and diarrhea. Autopsy showed extensive crystalline deposits in the renal parenchyma and pelvis and a marked recent bloody exudation in the peritoneal cavity. No mention was made of perirenal hemorrhage, but it is possible that the bloody exudate found represented such a hemorrhage since the animal had renal obstruction due to sulfonamide crystals. With this possible exception, the experimental[7,8] and clinical[9-13] administration of phthalylsulfathiazole has not been shown to cause bleeding in any

tissue. Poth[6] quotes Allen to the effect that one-fifth of his patients with carcinoma of the colon had increased bleeding on treatment with succinylsulfathiazole, but he reported that this did not occur following phthalylsulfathiazole.

The observations reported here suggest that the clinical administration of phthalylsulfathiazole should be avoided in the presence of diminished renal function due to ureteral obstruction.

Summary. The ureters were ligated in 6 dogs. and phthalylsulfathiazole was administered orally in doses of 0.5 to 0.75 g/kg/day for 3-6 days pre-operatively and post-operatively until death. Two of these animals showed at autopsy an increased vascularity of the fascial sheath covering the perirenal fat, 5 animals showed perirenal sheath hemorrhage, and 3 animals had fresh free blood in the abdominal cavity.

The ureters were ligated in 6 control dogs, and they received no phthalylsulfathiazole. Four of these animals showed at autopsy an increased vascularity of the perirenal sheath. None of the animals showed perirenal hemorrhage.

It was found that both ureter ligation and phthalylsulfathiazole administration are necessary for production of the perirenal lesions. It appears that the perirenal hemorrhage is due to vascular changes since no abnormalities in prothrombin time, bleeding, clotting, and clot retraction time, red blood cell counts or platelet counts were demonstrated. The average total blood sulfathiazole concentration at the time of death in the test animals was 2.9 mg %.

[6] Harrison, T. R., and Mason, M. F., *Medicine*, 1937, **16**, 1.

[7] Mattis. P. A., Benson, W. M., and Koelle, E. S., *J. Pharm. and Exp. Therap.*, 1944, **81**, 116.

[8] Poth, E. J., and Ross, C. A., *Texas Rep. Biol. and Med.*. 1943, **1**, 345.

[9] Poth, E. J., *Surgery*, 1945, **17**, 773.

[10] Poth. E. J., *Texas Rep. Biol. and Med.*, 1946, **4**, 68.

[11] Streicher, M. H., *J. A. M. A.*, 1945, **129**, 1080.

[12] Angelo, G., *Am. J. Surg.*, 1945, **70**, 354.

[13] Bargen, J. A., *Proc. Staff Meet., Mayo Clin.*, 1945, **20**, 85.

16086

Complement Fixation Studies in Experimental Histoplasmosis.

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Chiefly because of the absence of a specific, potent antigen, no satisfactory complement-fixation test has been recorded for histoplasmosis. Van Pernis, Benson and Holinger[1] conducted complement-fixation tests on a human patient with histoplasmosis, but obtained negative results. The antigens they used were (a) the undiluted filtrate from a dextrose broth culture medium inoculated with *H. capsulatum* and (b) the acetone precipitate redissolved in saline.

However, antigens have been prepared and successfully used for complement-fixation tests in other mycotic diseases. For example, Martin[2,3] employed antigens derived from the yeastlike phases of *B. dermatitidis* and *Candida sp.* for the fixation of complement in the presence of homologous antisera, whereas Martin,[4] Fonseca[5] and Smith[6] derived their complement-fixation antigens from the mycelial phase in their studies on chromoblastomycosis, South American blastomycosis and coccidioidomycosis respectively.

Since the causative organism of histoplasmosis appears in the yeast-like form in infected animals and human beings, an antigen derived from this phase might be expected to produce the most satisfactory serologic tests for this disease. After the yeast-like form of *H. capsulatum* was successfully grown in a semi-fluid medium in large quantities,[7] serologic studies were initiated. The purposes of these investigations were (1) to determine the binding power and specificity of the yeastlike phase of *H. capsulatum* as a complement-fixation antigen, and (2) to obtain information on the time of appearance, the maximum titer and the persistence of complement-fixation antibodies in animals infected with histoplasmosis. In this paper the results of these investigations are presented.

Materials and Methods. a. Complement-fixation antigens. The yeastlike phase of *H. capsulatum* was grown at 37°C in semi-fluid medium[7] for 3 weeks, centrifuged, washed in Tyrode's solution at pH 7.0, and killed by 0.5% formalin. The cells were then centrifuged at 32-36°F, washed twice in Tyrode's solution, and stored at 2-5°C. The antigen employed in the complement-fixation tests consisted of whole washed formalin-killed cells of the yeastlike phase, and will henceforth be referred to as "YP antigen." Although all lots had approximately equal potency, one lot of YP antigen (derived from strain 6521) was used in the following experiments. The antigenic titer was 1:256 against rabbit immune serum and the anticomplementary titer was 1:4-1:8. This antigen exhibited similar binding power in the presence of antisera against the homologous strain and against five other strains of *H. capsulatum*.

Histoplasmin is the broth filtrate from the growth of the mycelium of *H. capsulatum* on a modified Long's medium.[8] The fungus was grown at room temperature in the dark, with samples removed periodically for assay. During approximately the first 3 weeks of growth, the filtrate had no binding power in the presence of homologous antiserum. However, for the next 20-30 days a marked rise in binding power of the filtrate appeared (Fig. 1). Thereafter the titer remained at a fairly con-

1 Van Pernis, P. A., Benson, M. E., and Holinger, P. H., *J. A. M. A.*, 1941, **117**, 436.

2 Martin, D. S., *J. Infect. Dis.*, 1935, **57**, 291.

3 Martin, D. S., *Am. J. Trop. Med.*, 1942, **22**, 295.

4 Martin, D. S., *Am. J. Trop. Med.*, 1938, **18**, 421.

5 da Fonseca, O., *An. brasil. de dermat. e sifil.*, 1939, **14**, 112.

6 Smith, C. E., personal communication.

7 Salvin, S. B., *J. Bact.*, 1947, **54**, 655.

8 Emmons, C. W., Olson, B. J., and Eldridge, W. W., *Pub. Health Rep.*, 1945, **60**, 1383.
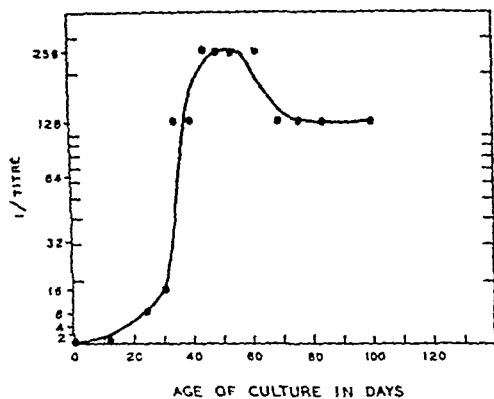
FIG. 1.

Development of binding power of histoplasmin from strain 6510 of *Histoplasma capsulatum* against homologous antiserum.

stant level, although there was a slow but distinct increase in the anticomplementary action as the culture aged, especially after the first 90 days of growth. For the experiments in which histoplasmin was used, a 60-day-old filtrate of strain 6510 was employed as the antigen.

b. *Complement-fixation test.* The method was that employed by Bengston.[9] Complement was titered in the presence of antigen each day tests were conducted and antigens were tested whenever new lots were employed. Four units of antigen, 2 of complement, and 1½ of amboceptor were used throughout the studies.

c. *Strains of fungi.* The fungi were from the collection in this laboratory. The yeastlike phase on which most of the studies were conducted was from a strain (No. 6521) from a naturally-infected dog.[10] The histoplasmin employed was produced by a culture of strain 6510.[11] Blastomycin was obtained from a broth culture of strain 6014 of *B. dermatitidis* isolated by Conant; and the coccidioidin, partly from a broth culture of strain 6210 of *C. immitis* isolated by Emmons, and partly supplied by C. E. Smith.

d. *Animals.* The experimental animals were adult albino rabbits, which were bled and their sera tested for complement-fixation antibodies in all cases with the appropriate antigens before inoculation. Among 228 animals tested, only 2 rabbits had sera that fixed complement in the presence of histoplasmin, and none in the presence of YP antigen. What caused these two rabbit sera to fix complement in serum dilutions of 1:8 and 1:16 was not learned, since no gross abnormalities were discernible. However, the two were discarded.

Experiments and Results. a. *Production of complement-fixation antibodies.* Fifteen rabbits were inoculated intravenously with a suspension of the live yeastlike phase of strain 6521 of *H. capsulatum.* They were bled from the ear veins periodically, and their serum assayed for complement-fixing antibodies with YP antigen.

For about 10 days after inoculation, no antibodies were demonstrable (Fig. 2). The antibody titer then began to increase sharply until by about the 30th day the antibody titer reached 1:512 for most of the animals. All rabbits developed antiserum that fixed complement in the presence of YP antigen. A rapid decrease in titer occurred for the 20 ensuing days, after which the titer remained more or less constant until the experiment was terminated 170 days after inoculation.*

When these same antisera were tested with histoplasmin as the antigen, much lower antibody titers were obtained (Fig. 2), the highest being 1:128 in comparison with the 1:512 with YP antigen. In addition, 7 of the 15 animals showed no positive titers at any time, in contrast to the positive titers demonstrated in all the animals with the YP antigen. With histoplasmin as the complement-fixation antigen, the maximum titer was attained during the third week after inoculation, after which the antibody titer decreased progressively until the eighth week, when no antibodies could be demonstrated.

b. *Reactions of H. capsulatum antigens with other fungal antisera.* (a) *Coccidioidomycosis.* YP antigen and histoplasmin were

9 Bengston, I. A., *Pub. Health Rep.,* 1944, 59, 402.

10 Olson, B. J., Bell, J. A., and Emmons, C. W., *Am. J. Pub. Health,* 1947, 37, 441.

11 McLeod, J. H., Emmons, C. W., Ross, S., and Burke, F. G., *J. Pediat.,* 1946, 28, 275.

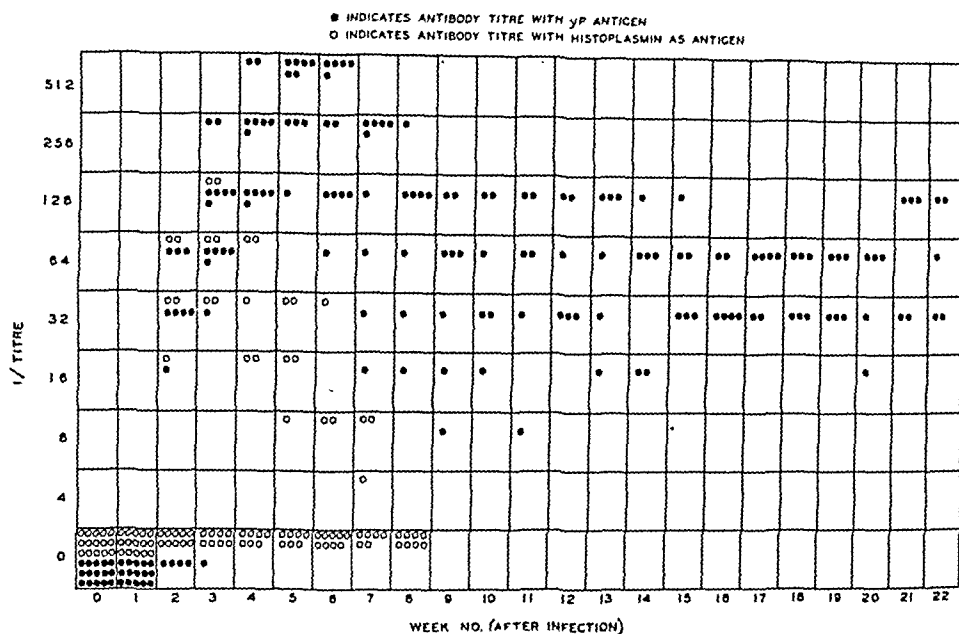* Not all the rabbits were bled on each "bleeding day."

FIG. 2.

Development of antibody titer of experimentally infected rabbits in the presence of (a) YP antigen and (b) histoplasmin.

Note: The decrease in the number of readings per week is due to the deaths of an increasing number of animals.

studied in complement-fixation tests with antisera from 8 rabbits experimentally infected with *C. immitis* and from 10 human patients with localized or disseminated coccidioidomycosis.[†] All the rabbits infected with *C. immitis* developed potent complement-fixation antibodies in the presence of the homologous antigen. However, at no time did YP antigen fix complement in the presence of coccidioidomycosis antibodies, although histoplasmin did, both with the rabbit and human antisera.

(b) *Blastomycosis.* Eight rabbits, inoculated intravenously with the living yeastlike phase of *B. dermatitidis*, were bled periodically and their antisera analyzed for the presence of complement-fixation antibodies with blastomycin as antigen. Antibodies were demonstrated about 20 days after infection, and remained until the ninth week. Throughout this period of demonstrable antibody with the homologous antigen, YP antigen produced no complement-fixation with the blastomy-

cosis antisera at any time. However, histoplasmin showed reactions with these heterologous antisera at the low titers of 1:8 and less.

(c) *Moniliasis.* Four rabbits were immunized by daily intravenous injections of formalin-killed cells of the budding phase of *Candida albicans.* The resulting antisera, active with the homologous antigen, were analyzed for complement-fixing properties in the presence of histoplasmin and YP antigen. Histoplasmin produced complement-fixation in antiserum dilutions as high as 1:8, whereas YP antigen showed no positive reactions whatsoever.

Discussion. Antigens from the yeastlike phase of *H. capsulatum* show promise as agents for determining the presence of complement-fixation antibodies, since these antigens have specificity, good binding power, and low anticomplementary titers with experimentally produced rabbit antisera. When histoplasmin was used as antigen, the titers were lower, of shorter duration, and less specific than with the YP antigen. Also of

† Thanks are expressed to Dr. C. E. Smith for providing these antisera.

interest was the persistence of a positive titer in the presence of YP antigen in the sera of rabbits with histoplasmosis after gross external symptoms of the disease had disappeared and blood cultures were no longer positive.

During the course of these studies on laboratory animals, two human patients appeared from whom *H. capsulatum* was isolated.‡ Their sera fixed complement in the presence

‡ Thanks are expressed to Drs. F. C. Kelly and J. Wells for making these sera available.

of YP antigen in dilutions of 1:32 and 1:128. More than 30 "normal" persons from whom *H. capsulatum* was not isolated had no complement-fixing antibodies in the presence of YP antigen.

Summary. An antigen from the yeastlike phase of *Histoplasma capsulatum* fixed complement in the presence of antisera from experimentally infected rabbits from the 2nd to the 23rd weeks following infection. In contrast to histoplasmin, this antigen did not react with several other fungal antisera.

16087

Sulfactin. Bacterial Spectrum, Toxicity and Therapeutic Studies.*

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A new antibiotic, sulfactin, has been described by Junowicz-Kocholaty, Kocholaty, and Kelner.[1] A description of the actinomyces, the method for growing the organism, and the purification and chemical properties of the antibiotic substance were given by the above authors. It is the purpose of this publication to present results of studies on the bacterial spectrum and the toxicity and therapeutic action in mice.

Experimental. The sterile crude culture filtrate of Actinomyces, R-30, was employed for determining the bacterial spectrum. In general, the *in vitro* antibacterial tests were carried out at the time similar tests were being made with actinorubin and lavendulin employing Bacto-nutrient broth adjusted to pH 7.3 and by the technic described previously by Kelner and Morton.[2] Since sulfactin is more inhibitory for gram-positive than for

[1] Junowicz-Kocholaty, R., Kocholaty, W., and Kelner, A., *J. Biol. Chem.*, 1947, **168**, 765.

[2] Kelner, A., and Morton, H. E., *J. Bact.*, 1947, **53**, 695.

gram-negative organisms, it was standardized against *Staphylococcus aureus*, P210. The smallest amount of sulfactin per ml of Bacto-nutrient broth, pH 7.3 which prevented growth of *S. aureus*, under the conditions of the test, was designated as one dilution unit.

The crude culture filtrate of Actinomyces, R30, was titrated against *S. aureus*, P210, in Bacto-nutrient broth, pH 7.3, and in the same medium to which had been added 0.5% NaCl. In each medium the activity titrated to the same tube, representing 6,400 dilution units. Thus the antibacterial action of sulfactin is not appreciably reduced in the presence of 0.5% NaCl. The presence of 10% sterile, defibrinated, normal horse blood in the Bacto-nutrient broth with salt necessitated 10 times more sulfactin to inhibit *S. aureus*.

There was received on June 20, 1946, from Dr. Junowicz-Kocholaty about 60 mg of recrystallized sulfactin which had been dried over $CaCl_2$. By the agar streak technic of assay it was estimated that 0.0254 µg of this material equalled one unit. The material was put in solution as follows: To 10.43 mg of sulfactin was added one ml of chloroform and warmed to 45°C until dissolved. One ml of

TABLE I.
Results of *in vitro* Antibacterial Tests with Sulfactin.

Organism	Strain	Amt of sulfactin/ml of medium inhibiting the strain
Aerobacter aerogenes	P41	>32
Alcaligenes faecalis	P61	>32
Brucella abortus*	P62, P18A	>32
" *melitensis**	P80	>32
" *suis**	P64	>32
Chromobacterium violaceum	P104	>32
Eberthella typhosa	P11, P115	>32
Escherichia coli	P216	>32
" *communior*	P218	>32
" *neapolitania*	P161	>32
Klebsiella pneumoniae	ATCC No. 8050	>32
Mycobacterium smegmatis	P49	>32
Neisseria catarrhalis	P66	>32
Proteus vulgaris	P98, P188	>32
Pseudomonas aeruginosa	P2, P186A	>32
Salmonella enteritidis	P51	>32
" *paratyphi*	P198A	>32
" *schottmuelleri*	P35	>32
Sarcira lutea	P6	>32
Serratia marcescens	P4	>32
Shigella dysenteriae	P52A	>32
" *paradysenteriae*, Flexner	P21	>32
Streptococcus faecalis†	P122	>32
Trichophyton interdigitale		>32
Vibrio comma	P215	>32
Alpha streptococcus†	P26	32
Gamma streptococcus†	P169	32
Bacillus anthracis	P119	16
" *cereus*	P109	16
" *mycoides*	P34A, P58A	16
" *anthracis*	P60	8
Gaffkya tetragena	P140A	8
Bacillus subtilis	P219	4
Streptococcus pyogenes†	P24	4
Bacillus circulans		2
" *megatherium.*	P97	2
" *mycoides*	P33A	2
" *subtilis*	P23A	1
Diplococcus pneumoniae, type 1†	P27	1
Staphylococcus aureus	P210	1
Bacillus mesentericus	P112	0.5
" *mycoides*	P156	0.5
" *subtilis*	P7	0.5
Diplococcus pneumoniae, type 3†	P29	0.5
Gaffkya tetragena	P43	0.5
Neisseria sicca	P141A	0.5
Micrococcus aurantiacus	P103	0.25
" *lysodekticus*		0.25
" *roseus*	P120B	0.125
Corynebacterium xerose	P82	0.06
" *diphtheriae*	P1	0.03

* The tests with *Brucella* were made in Bacto-tryptose broth.

† Tests with *Streptococci* and *Diplococci* were carried out in Bacto-nutrient broth, pH 7.3, to which had been added 0.5% NaCl and 5% sterile, normal defibrinated horse blood.

95% ethyl alcohol was added then distilled water to make 10 ml. When assayed by the technic employing broth, the material appeared considerably more active than when assayed by the agar streak technic. In broth 0.0048 μg of sulfactin per ml inhibited *S.* *aureus*. It was found that a concentration of 0.0097 μg per ml of Bacto-nutrient broth to which had been added 0.5% NaCl and 5% sterile, normal, defibrinated horse blood prevented visible growth of *Diplococcus pneumoniae*, type 1, P28 for 48 hours at 37°C.

TABLE II.
The Toxicity of Sulfactin for White Mice by Intraperitoneal Injection.

Dose of sulfactin	No. of mice injected	No. of mice living	No. of mice dying
0.1 mg	5	5	0
0.5	5	5	0
1.0	5	4	1*
5.0	4	0	4†

* The animal was found dead on the morning of the 8th day. A gram-negative rod was isolated from the heart's blood. The tissues were too badly autolyzed for a critical pathological examination.

† All animals died within 3.5 hours after injection. All animals showed a reaction within 2 minutes after injection; one mouse had violent tremors. It was not entirely satisfactory working with concentrations of 5 mg of sulfactin per ml of water because of the insolubility of the product at that concentration. The sulfactin was put into solution with chloroform at 45-50°C, distilled water added, and most of the chloroform evaporated by bubbling a small stream of sterile air through the liquid. This resulted in a heavy white suspension, relatively free of chloroform vapor. It is not certain that the early deaths of the mice receiving 1 ml of the suspension containing 5 mg of sulfactin can be attributed to the pharmacologic action of the drug. Nothing abnormal could be detected by gross examination, except, perhaps, that the liver and kidneys appeared a bit pale. Microscopic examination revealed that the capillaries in the lungs contained an excessive number of polymorphonuclear leukocytes. The pancreas, at the periphery of the lobes in 3 of the 4 mice, showed well marked degeneration of the outer zone of acini. Large numbers of fragmented nuclei were found in the "reaction centers" of the thymus and lymphoid follicles of the spleen. Other tissues appeared essentially normal. Pathological studies did not permit an opinion of the mechanism of death of the animals.

Because of the high virulence of our strain, this organism was selected for therapeutic studies.

The toxicity of sulfactin was determined by intraperitoneal injection into white mice weighing 17 to 20 g each. The results are summarized in Table I. By applying the formula of Reed and Muench[3] to the results, the LD_{50} is calculated to be about 2.75 mg. The necessity for employing the LD_{50} is set forth in the footnotes to Table I.

The mice which survived the injections of sulfactin were sacrificed for pathological studies. Two mice which received one mg each of sulfactin were sacrificed 14 days after the injection of the drug. Possible slight enlargement of the liver was the only gross pathological c h a n g e detectable. These changes were in no ways as extensive as those observed in mice following the injection of 0.75 mg of actinorubin.[4] Two mice which received 0.5 mg each of sulfactin were sacrificed 14 days after the injection and a possible enlargement of the liver was the only gross pathological change observable. Two mice which received 0.1 mg each of sulfactin

3 Reed, L. J., and Muench, H., Am. J. Hyg., 1938, 27, 493.

4 Morton, H. E., Proc. Soc. Exp. Biol. and Med., 1947, 64, 327.

were sacrificed 14 days after the injection and no gross pathological changes were detectable.

Twenty-one days post-injection 2 mice which had received one mg, 2 mice which had received 0.5 mg and 2 mice which received 0.1 mg sulfactin each were sacrificed. Nothing abnormal could be observed microscopically. None of the organs of the mice sacrificed 14 days and 21 days post-injection showed anything abnormal by microscopic examination. We are indebted to Dr. Herbert Ratcliffe for the pathological studies on these animals.

For a preliminary test of the therapeutic potentialities of sulfactin it was tested against *D. pneumoniae*, type I in mice. Serial dilutions of a 24-hour-old blood broth culture of the test organism were made in nutrient broth. One ml portions of the various dilutions of the test organism were inoculated intraperitoneally into mice to determine its pathogenicity, and one ml of each dilution was made into a poured blood agar plate to estimate the number of organisms constituting a lethal dose. Those mice receiving sulfactin received it intraperitoneally within a few seconds after the injection of the culture. The results are summarized in Table II. The results of the protection test made on July 2nd appeared so promising that a second ex-

TABLE III.

Ability of Sulfactin to Protect Mice Infected with *Diplococcus pneumoniae*, Type 1.

Diln. of culture, 1 ml/mouse	No. of organisms dose*	No. of mice inj.	Amt of sulfactin inj.	No. of mice living	No. of mice dying
		Experiment of July 2, 1946.			
10-5	∞	5	0	0	5
10-6	284	5	0	0	5
10-7	28	5	0	0	5
10-8	5	5	0	0	5†
10-9	0	5	0	3	2†
10-5	1000 M.L.D.	5	0.1 µg	0	5†
10-5	,, ,,	5	0.5 ,,	0	5†
10-5	,, ,,	10	1.0 ,,	5	5†
10-5	,, ,,	5	2.0 ,,	1	4‡
10-5	,, ,,	5	10.0 ,,	5	0
		Experiment of July, 10, 1946.			
10-5	∞	5	0	0	5
10-6	>300	5	0	0	5†
10-7	51	5	0	0	5
10-8	8	5	0	1	4†
10-9	1	5	0	4	1†
10-10	1	5	0	5	0
10-5	<1000 M.L.D.	10	0.5 µg	0	10†
10-5	,, ,,	10	1.0 ,,	0	10†
10-5	,, ,,	10	5.0 ,,	4	6†
10-5	,, ,,	10	10.0 ,,	6	4†

* No. of organisms estimated by inoculating 1 ml, the same amount as was injected into each mouse, into a poured blood agar plate.

† *D. pneumoniae* isolated from the heart's blood at autopsy.

‡ Three mice died between the 2nd and 3rd day after injection and pneumococci were isolated from the heart's blood of each. One mouse was found dead in the cage during the 6th day with its nose chewed. The heart's blood culture was sterile, so it is doubtful that it died of pneumococcal infection.

periment was made eight days later. In the first experiment the amount of sulfactin which protected 50 per cent of the mice against 1,000 minimum lethal doses of culture was in the order of 1 or 2 µg. This compares quite favorably with the LD_{50} toxic dose of about 2.750 µg. In the second experiment the challenging dose of culture may have been slightly less than 1000 M.L.D. The sulfactin was another lot prepared from the original purified material. The amount of sulfactin which protected against nearly 1000 M.L.D. of culture was of the order of 7.5 µg. This is still a quite favorable comparison to the toxic dose. There was not sufficient material avail-

able for further toxicity and therapeutic studies.

Studies on drugfastness and comparison with penicillin in this respect have been made and will be reported later.

Summary. Sulfactin is active primarily against gram-positive microorganisms. The LD_{50} for mice of 17-20 g by intraperitoneal injection is about 2,750 µg. The amount of sulfactin required to protect mice against the intraperitoneal injection of approximately 1000 minimum lethal doses of *Diplococcus pneumoniae*, type 1, is of the order of 1 to 7.5 µg.

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Experimental Type Transformation of *Shigella paradysenteriae* (Flexner).

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Griffith and Dawson observed type transformation of pneumococci *in vivo* and *in vitro* induced by dead pneumococci or extracts of the transforming type. The transforming principle was shown by Avery and his collaborators to be a desoxyribonucleic acid (for references see[1]). Recently, Boivin[2,3,4] reported transformation of an *E. coli* strain induced by a filtrate strain of a different type. Again the inducing agent was identified as a desoxyribonucleic acid. The theoretical aspects of these observations have been discussed.[5]

Considerable information obtained in recent years on the antigenic properties of Shigellae, particularly *Shigella paradysenteriae* (Flexner)[6,7] provide a basis for an investigation of type transformation of *Shigellae*. During the last 2 years several attempts were made to obtain evidence of this kind. The first results were disappointing, but information worthwhile mentioning here was obtained: we found it difficult to produce true R variants of Flexner bacilli on a number of fluid and solid media, including synthetic ones[8] providing only minimal requirements for growth. Cultivation in homologous immune serum in concentrations up to 10% was also not conducive to the appearance of R variants. Bacteria grown in broth containing up to 10% of homologous or heterologous immune serum or in normal serum gave no indications of changes in their antigenic behavior. Also addition of formol-killed bacteria to nutrient broth of a heterologous type did not influence the cultural and serological properties of *Shigellae*. However, in the light of experiences reported below it cannot be excluded that the number of experiments with this technique was insufficient to disprove the possibility of obtaining type transformation with killed organisms as inducing agent.

However, we have been able to secure evidence of type transformation in Flexner bacilli with extracts similar to those employed by Boivin.

Technique. Cultures of *Sh. paradysenteriae* were grown in beef heart broth (without dextrose, pH 7.4) at 37°C for approximately 18 hours. Ten drops of toluene were added to each 40 ml of culture, which was kept at room temperature for 4 hours under repeated shaking. The bacteria were separated by centrifugation in an angle centrifuge placed in a chillroom (±4°C). The supernatant fluids of two 40 ml tubes were pooled and sent through a filter candle of medium porosity. The filtrates were stored in the chillroom and distributed in 3 ml volumes in small test tubes.

The filtrates were inoculated from cultures grown 18 hours at 37°C on beef heart agar slants. After incubation at 37°C overnight platings on beef heart agar were made. They were repeated one or several days later as desired. Sterility tests accompanied each step of the procedure, and an uninoculated tube was incubated and plated out together with the inoculated filtrates.

Strains from our collection whose stability in serological and cultural properties was known for at least one year were employed. They were examined anew before entering the experiments.

[1] McCarty, M., *Bact. Rev.*, 1946, 10, 63.

[2] Boivin, A., Delaunay, A., Vendrely, R., and Lehoult, Y., *Experientia*, 1945, 1, 334.

[3] Boivin, A., Delaunay, A., Vendrely, R., and Lehoult, Y., *Experientia*, 1946, 2, 139.

[4] Boivin, A., and Vendrely, R., *Helvet. chim. acta*, 1946, 5, 1338.

[5] Luria, S. E., *Bact. Rev.*, 1947, 11, 1.

[6] Weil, A. J., Black, J., and Farsetta, K., *J. Immunol.*, 1944, 49, 321 and 341.

[7] Weil, A. J., *J. Immunol.*, 1947, 55, 363.

[8] Weil, A. J., and Black, J., PROC. SOC. EXP. BIOL. AND MED., 1944, 55, 24.

Colonies isolated from platings were examined by the slide agglutination technique with absorbed sera.[6,7] If mutants were obtained, the colonies and, at the same time, non-mutated colonies were secured on agar slants and compared in test tube agglutinations and in cultural tests with the behavior of the parent strain and that of the strain from which the filtrate was derived ("inducing strain"). The following cultural methods were used in these comparative tests: beef heart agar for the observation of growth, colony form and microscopy, beef heart broth incubated both at room temperature and at 37°C, from which hanging drops were examined, appropriate media for tests of indol formation and of reduction of trimethylamine oxide, the decomposition of urea, the liquefaction of gelatin, and formation of acid and gas from dextrose, lactose, mannitol, maltose, dulcitol, rhamnose, arabinose, and salicin.

Filtrates were usually inoculated in groups of 6 with the corresponding strains, thus adding an additional safeguard by cross check. Altogether 38 filtrates involving Types I, II, II.VII, III, V, VI, VII, VIII, IX, and XII of the Flexner bacillus* were inoculated with at least 6 strains of different types. In 225 tests unequivocal evidence of type transformation was obtained in 3 cases, namely a filtrate of Type I inoculated with a strain of Type II.VII, a filtrate from a strain Type II inoculated with a strain of Type V, and a filtrate of a different strain Type II inoculated with a strain Type XII. In each case platings showed colonies both of the parent type and of the type of the inducing filtrate. In the first case, of 16 colonies examined, 10 were of Type II.VII and 6 of Type I; in the second case, of 40 colonies, 12 were of Type V and 28 were of Type II; and in the third case, of 95 colonies 49 were of Type XII and 46 of Type II. These figures were obtained from a single plating in the first case, and from 3 platings made on different days in the second and third case.

The Type V strain of the second case of transformation differed from the inducing strain of Type II, in that the Type V strain

was indol positive whereas the Type II strain did not form indol. All colonies from the platings reacting serologically like the parent strain gave a positive indol reaction, whereas the mutated colonies had lost the ability to form indol. As it happened, the parent and the inducing strain differed in a second property namely, the Type V strain does not form acid from maltose, whereas the inducing Type II strain acidifies this carbohydrate within 24 hours. Both non-mutated and mutated colonies from the platings did not acidify maltose. We then have here a mutation of one biochemical property coupled with the transformation of the somatic antigen, whereas a second property remains unchanged, thus tagging the mutants according to their parentage.

In the 2 other cases of transformation, the parent and the inducing strain were indistinguishable in their cultural properties and therefore did not offer a chance to obtain additional data on the association of mutation of biological and serological properties.

The parent Type II.VII strain of the first mentioned case of transformation grows on agar in slightly more opaque colonies than those of the inducing Type I strain. In the cultures obtained from the filtrates, those of serological Type II.VII were more opaque than the mutant colonies of Type I. This is illustrated in Fig. 1 where the parent and the inducing strain and one non-mutated and one mutated culture were plated out on separate quadrants of an agar plate. The difference in opacity may conceivably be merely the visible expression of the change in antigens. But it is also possible that opacity depends on some physical property of the bacteria independent from the somatic antigen. There is at present no way to decide whether or not the differences in opacity indicate another associated mutation.

The mutant strains have now been under observations for 10, 8, and 2 months respectively. During this period they were repeatedly plated out and numerous single colonies were tested for evidence of reversion to the original type. No such evidence was obtained.

Hitherto we have been unable to reproduce
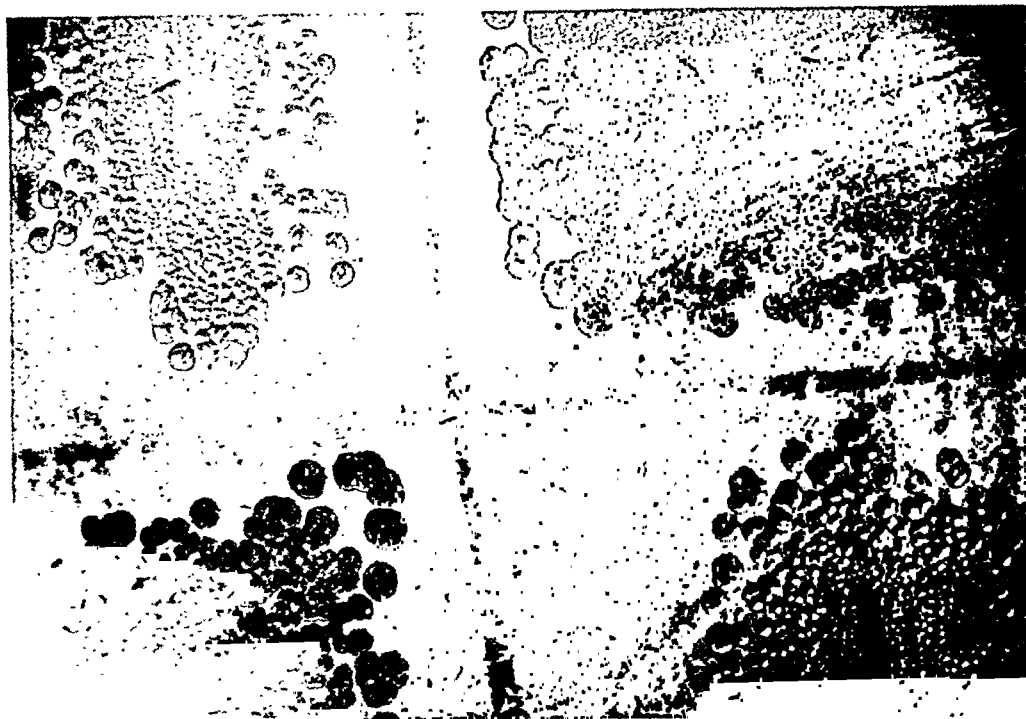
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* For type designation see [6].

FIG. 1.

Upper right: parent strain (type II.VII).
Upper left: inducing strain (type I).
Lower left: subculture from non-mutated colony (type II.VII).
Lower right: subculture from mutated colony (type I).
Note: The apparent paleness of some of the upper left hand section is due to the circumstance that this is the point of maximal illumination.

at will the phenomenon described. That is, new filtrates made from the inducing strains did not evoke mutations from the strains with which we were successful the first time.

Discussion. In 3 out of 225 attempts evidence of induced type transformation of Flexner bacilli by culture filtrates was obtained. In the single case where the parent and the inducing strain were distinguishable in their cultural behavior, one such distinguishing property, namely that of indol formation, was coupled to the change in somatic antigen, whereas a second one, namely acid formation from maltose, was not correlated with the serological transformation. Our experience resembles that of Boivin[2,3,4] in two important points. Induced transformation could be produced in relatively rare instances —a single one in Boivin's case and 3 in ours. Moreover, Boivin found a mutation in a bio-

chemical capacity (sucrose fermentation) related with the change in antigen just as we found the serological mutation associated with the property of forming indol.

In our experience, the type transformation was not visibly mediated by a transition into the R phase. The lack of visible evidence of the R phase preceding transformation may, but does not necessarily, indicate a difference in the mechanism of transformation of *Sh. paradysenteriae* and that of *E. coli* and pneumococcus. Further study of this point is clearly indicated.

We were less fortunate than Boivin in that we have not been able to reproduce transformation at will with a given filtrate and a given strain, while Boivin evidently could, as indicated by the fact that he could collect amounts of filtrates large enough to proceed with an isolation of the desoxyribonucleic

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Hitherto we have been unable to reproduce

* For type designation see [6].

TABLE I.
Individual Data and Results of the Pregnancy Test in Mares and Female Donkeys.

Case No.	Age (years)	Wt (kg)	Days after insemination	Hyperemia reaction	Remarks
A. Mares					
1	9	501	90	Positive	Parturition, 330 days pregnant
2	13	470	78	,,	Not pregnant*
3	12	400	80	,,	Parturition, 327 days pregnant
4	15	336	72	,,	,, 328 ,, ,,
5	13	415	88	,,	,, 341 ,, ,,
6	18	343	68	,,	Not pregnant*
7			72	,,	Pregnant
8			56	Negative	Not pregnant
9			42	,,	,, ,,
10			78	Positive	Parturition, 327 days pregnant
11	15	370	80	,,	Pregnant
12	9	370	71	,,	,,
13	13	420	81	Negative	Not pregnant
14	6	430	83	,,	,, ,,
15	10	450	69	Positive	Pregnant
16		400	62	Negative	Not pregnant
17	16	400	62	,,	,, ,,
18	5	330	76	Positive	Pregnant
19	11	390	78	,,	,,
20			87	,,	,,
21	12	410	85	Negative	,, †
22	16	460	76	,,	Not pregnant
23	9	340	64	Positive	Pregnant
24			114	Negative	Not pregnant
25			138	Positive	Parturition, 350 days pregnant
26			138	,,	,, 310 ,, ,,
27			138	,,	,, 335 ,, ,,
28	11	473	74	,,	Not pregnant*
29	4	300	71	,,	Pregnant
30	6	450	69	,,	,,
31	7	490	75	Negative	,, †
32	5	255	57	,,	Not pregnant
33	6	345	70	Positive	Pregnant
34	5	305	73	,,	,,
35	6	408	63	Negative	,, †
36	13		65	,,	,, †
37	9	390	71	,,	Not pregnant
38	7	370	61	Positive	Pregnant
B. Female donkeys					
1			75	Negative	Not pregnant
2			128	,,	,, ,,
3			133	Positive	Parturition, 368 days pregnant
4			113	,,	,, 357 ,, ,,

* False-positive tests.
† False-negative tests.

the blood sample. The clinical condition of the animal agreed with the biological test (both positive and negative reactions) in 35 cases. It did not agree in 7 instances. Four mares definitely pregnant gave negative test 85, 75, 65 and 63 days after fertilization. On the other hand, 3 non-pregnant mares gave positive test 78, 74 and 68 days after insemination.

Bunde[5] pointed out that in human pregnancy, by employing the same hyperemia test, there occurred only false-negative test, i.e.

negative reaction with urine of pregnant women. In equids, however, there occurred both types, namely negative test with serum of pregnant mares and positive test with serum of non-pregnant ones. The false-negative tests are difficult to explain for, according to the data of Day and Rowlands,[7] the high circulating level of gonadotrophin in mares occurs between the second and the fourth month of

[7] Day, F. T., and Rowlands, I. W., J. Endocrinology, 1940, 2, 255.

acid. Thus, the factors involved in the induction of type transformation of Flexner bacilli require further study both concerning the parent organisms and the properties of filtrates.

The demonstration of induced type transformation in *Shigellae* is not only of theoretical interest,[5] but it also cannot fail to influence our viewpoints on problems of the biology of the enteric flora. These have just been discussed at some length,[7] and it may suffice here to recall this aspect of induced type transformation.

Summary. Induced type transformation of *Sh. paradysenteriae* (Flexner) has been obtained in 3 out of 225 experiments. The inducing principle is contained in filtrates of broth cultures.

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Ovarian Hyperemia in the Immature Rat as a Pregnancy Test in Equids.

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Early diagnosis of pregnancy in equids by means of a biological reaction was studied by Cole and Erway.[1] A new method has been introduced by Zondek and Sulman[2] based on the ovarian hyperemia in immature rats injected with serum gonadotrophin. The same reaction proposed by Salmon *et al.*,[3] as a 6-hour test for human pregnancy, was challenged by Farris[4] and accepted with restrictions by Bunde.[5] Recently Zondek and Sulman[6] have recommended it again as a 24-hr test for the diagnosis of equine pregnancy. This note deals with the evaluation of the new test which was employed as a preliminary step for the preparation of mare gonadotrophin.

Experimental. Blood samples from 38 mares and 4 female donkeys obtained between 42 and 138 days after natural insemination were tested. Two infantile female rats weighing 40-55 g were injected subcutaneously with 1 and 2 cc respectively of each serum and killed 24 hours later. The ovaries were inspected in daylight and the response considered positive when they were enlarged and their color matched that of the kidney or the spleen. Pale yellow and small sized ovaries were indications of a negative response. Occurrence of pregnancy was assumed when one or both of the injected rats showed a positive response. With this technique and using rats from the same colony, positive reaction was always obtained 24 hours after the subcutaneous injection of 10 I.U. of a standard preparation of equine gonadotrophin. Therefore, when a positive response with 2 cc of serum was obtained, a blood concentration of at least 2,500 I.U. of gonadotrophin per liter was assumed to be present. Of course this is not an exact quantitative measurement but only a routine approximation.

A careful record was kept of each animal until evidence of pregnancy was established either by obvious abdominal enlargement or by subsequent parturition.

Results. Table I gives the results of the test with the serum of 38 mares and 4 female donkeys and the number of days between the natural insemination and the taking of

TABLE I

Effect of Ozone on Anti-A Isoagglutinin Titer.

Anti-A Isoagglutinating serum	Serum dilutions										
	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024
Untreated control	+++	+++	+++	+++	+++	+++	+++	++	+	±	—
T-4 min.	+++	—	+	±	—	—	—	—	—	—	—
T-8 "	—	—	—	—	—	—	—	—	—	—	—

+++, ++, +, ± = Varying degrees of agglutination of Group A erythrocytes.
— = No agglutination.
T = treated with ozonized oxygen.

TABLE II

Effect of Ozone on Anti-B Isoagglutinin Titer.

Anti-B Isoagglutinin serum	Serum dilutions										
	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024
Untreated control	+++	+++	+++	+++	+++	+++	++	+	+	+	—
T-4 min.	+++	+++	+++	+++	+++	±	—	—	—	—	—
T-8 "	+++	+++	+++	+++	++	—	—	—	—	—	—
T-10 "	—	—	—	—	—	—	—	—	—	—	—
T-14 "	—	—	—	—	—	—	—	—	—	—	—

+++, ++, +, ± = Varying degrees of agglutination of Group B erythrocytes.
— = No agglutination.

TABLE III

Effect of Ozone on Anti-A Titer of Ascaris Immune Serum.

Anti-A ascaris immune serum	Serum dilutions										
	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024
Untreated control	+++	+++	+++	+++	+++	++	+	+	+	+	—
T-10 min.	++	++	++	++	++	+	—	—	—	—	—
T-15 "	—	—	—	—	—	—	—	—	—	—	—

+++, ++, +, ± = Varying degrees of agglutination of Group A erythrocytes (human).
— = No agglutination.

pregnancy. It would be necessary to assume that in the 4 false-negative cases cited in Table I, the concentration of blood gonadotrophin, when the serum was tested, was less than 2,500 I.U. per liter, despite the fact that it was 63 to 85 days after definitely established fertilization. The false-positive tests, *i.e.* positive reaction with serum of non-pregnant mares, occurred in 11-, 13- and 18-year-old animals. It is possible that age may increase the blood level of gonadotrophin in equids more markedly than in postmenopausal women, but it is unknown to what extent.

Since positive or negative responses are unreliable in 17% of the cases, it seems that the ovarian hyperemia test in immature rats can be used only as a first approach to the exact diagnosis of pregnancy in equids.

Summary. The ovarian hyperemia reaction in immature rats was studied following the subcutaneous injection of equine serum from 38 mares and 4 female donkeys, 42-138 days after natural insemination. The accuracy of this 24-hour pregnancy test was 83%, failures being observed both by negative reaction in 4 pregnant cases and positive reaction in 3 non-pregnant ones.

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Effect of Ozonized Oxygen on Anti-A, Anti-B, and Anti-Rh Typing Sera.[*]

G. ALBIN MATSON AND CON FENNING.

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Reports of attempts to modify agglutinating and "blocking" antibodies against human erythrocytes have recently appeared in the literature. Boyd,[1] following the lead of Tyler,[2] reported on the effect of photo-oxidation on isohemagglutinins, anti-A and anti-Rh in the presence of Eosin Y as a sensitizer. Photo-oxidation produced by photoflood lights gradually destroyed the agglutinating activity but did not convert any of the agglutinins studied into "inhibiting," "incomplete," or "blocking" antibodies. An exposure of 15½ hours to these photoflood lights was sufficient to destroy completely the anti-Rh agglutinin. Anti-A agglutinin, however, required an exposure of 65 to 71 hours to destroy all agglutinating activity. Although it seems likely that the action of the photoflood lights in Boyd's experiments was that of oxidation, the possibility remains, nevertheless, that some other photo-dynamic effect may have played a part in modifying the agglutinins. It seemed desirable to us, therefore, to determine the effects on such agglutinins using a different oxidizing agent.

Materials and Methods. In this study ozone was employed to determine the effect of oxidation on isohemagglutinating antibodies, since this agent has useful attributes not possessed by other oxidizing agents. There are, for example, no added end products of the reactions other than those formed from the use of O_3. Ozone is a powerful oxidizing agent that will combine with unsaturated carbon atoms and form ozonides. The normal mode of action of ozone is solely an addition with no secondary oxidation. Furthermore, the hydrogen atoms attached to unsaturated centers are left undisturbed.

Two cc quantities of the selected sera were subjected to ozonized oxygen having a concentration of approximately one part ozone to 1000 parts of oxygen. Volume concentrations were ascertained by the iodine liberation technique in which free I is liberated from

1 Boyd, William C., *J. Exp. Med.*, 1946, **83**, 221.
2 Tyler, A., *J. Immunol.*, 1945, **51**, 157.

TABLE IV.
Effect of Ozone on the Titer of Anti-D (Rh₀) Typing Serum.

Rh₀ Agglutinating & blocking anti-body serum	2% Erythrocyte suspensions in saline	Serum dilutions in saline									
		1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
Untreated control Rh₀ Agglutinating serum	O Rh'	—	—	—	—	—	—	—	—	—	—
	O Rh"	+++	+++	+++	+++	+++	+++	+++	++	—	—
	O Rh₀	+++	+++	+++	+++	+++	+++	+++	—	—	—
T-4 min. Rh₀ agglutinating serum	O Rh'	—	—	—	—	—	—	—	—	—	—
	O Rh"	—	—	—	—	—	—	—	—	—	—
	O Rh₀	—	—	—	—	—	—	—	—	—	—

	2% Erythrocyte susp. in albumin	Serum dilutions in bovine albumin									
		1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
Control Rh₀ "Blocking" serum	O Rh'	+++	+++	+++	+++	+++	++	++	±±	—	—
	O Rh₂	+++	+++	+++	+++	+++	++	++	±±	—	—
	O Rh₀	+++	+++	+++	+++	+++	—	—	—	—	—
T-10 min. Rh₀ "Blocking" serum	O Rh'	+++	+++	+++	+++	++	+±	±±	—	—	—
	O Rh₂	+++	+++	+++	+++	++	+±	±±	—	—	—
	O Rh₀	+++	+++	+++	+++	++	—	—	—	—	—
T-20 min. Rh₀ "Blocking" serum	O Rh'	±±±	±±	—	—	—	—	—	—	—	—
	O Rh₂	±±±	±±	—	—	—	—	—	—	—	—
	O Rh₀	±±±	—	—	—	—	—	—	—	—	—

+++, ++, +, ± = Varying degrees of Group O Rh cells.
— = No agglutination.

KI by O_3 in an acidic solution. Iodine in turn was measured by titration with standard thiosulphate using starch as an indicator. Gas flow was regulated to one liter per 3 minutes of time. Calibration of flow before and after treatment of the specimen was done using a reliable gas meter.

The serum was placed in a 75 cc beaker in order to have ample space for the resulting foam, and the gas delivered by means of a drawn glass tip. The foam was agitated to make available new foam to the active agent.

The manufacture of the ozone from O_2 was accomplished with an electrical ozonizer developed by one of us (Fenning).[3] The output of a modulated 125 megacycle oscillator activated with D.C. pulses with a repetition frequency of 125/sec having a duration of 10 microseconds and a peak power of 2000 Watts was used to activate the ozonizer.

The anti-A and anti-B typing sera used in this work were obtained from group B and A subjects respectively who had been stimulated with group A and B substance by the method of Witebsky et al.[4] These sera were of high titer and avidity. Anti-A immune serum prepared by inoculating rabbits with saline extracts from the cuticle of Ascaris lumbricoides was also used in these tests. This anti-A immune rabbit serum was of high titer and reacted specifically against human group A erythrocytes but exhibited no titer against group B or O erythrocytes.

The Rh antiserum used was the anti-D (Rh_o) 85% specificity and was absorbed and processed in the Blood Grouping Laboratory in the Department of Bacteriology at the University of Utah. It had a titer of 1:128.

All erythrocyte suspensions used were of 2% strength in terms of blood sediment. They were washed once in physiological saline and resuspended. Physiological saline was used as the diluent of both sera and erythrocytes in all tests except those in which "blocking" serum was used. In these blocking tests bovine albumin, produced by Armour and Company,

was used as the diluent for both erythrocytes and serum.

Results and Discussion. Results of these tests are shown in the tables. From the data shown in Table I it is apparent that the group A isoantibody was partially destroyed in 4 minutes treatment with ozone and completely destroyed by 8 minutes of exposure.

A group A anti-B serum of identical titer and avidity similar to the anti-A serum was treated with ozone, and the results of this experiment are shown in Table II.

From the data presented in Table II it is apparent that the group B isoantibody was completely destroyed in 14 minutes and partially destroyed in lesser periods of time.

Anti-A immune serum produced in rabbits by inoculating them with cuticle from Ascaris lumbricoides was treated in a similar manner as were anti-A and anti-B isoagglutinin sera. The results of this experiment are shown in Table III.

The evidence in Table III indicates that the anti-A titer of Ascaris immune serum was lost following 15 minutes of exposure and partially lost during lesser periods of exposure.

The effect of ozone on anti-D (Rh_o) serum is shown in Table IV.

With reference to Table IV, it is apparent that progressive partial loss of titer was present in all sera tested. Furthermore, our observations show a complete loss of agglutinating and blocking antibody titer with suitably prolonged exposures in all sera of all types so far tested.

It may also be observed that whereas the "bivalent," "heat labile," "agglutinating," "complete," D (Rh_o) antiserum was destroyed by 4 minutes treatment it took 20 minutes of the same treatment to inactivate the "univalent," "heat stable," "blocking," "incomplete," D (Rh_o) antibody. These results are in agreement with the observations of other authors[5,6] with respect to the action of physical factors such as temperature and pressure on these 2 kinds of antisera.

3 Demonstrated A.A.A.S. exhibit, Dec., 1946, Boston, Mass.

4 Witebsky, E., Klendshoj, N. C., and McNeil, C., Proc. Soc. Exp. Biol. and Med., 1944, 55, 167.

5 Diamond, Louis K., *J. Lab. and Clin. Med.,* 1945, 24, 122.

6 Boyd, William C., *J. Exp. Med.,* 1946, 83, 401.

The question still remained as to whether treatment with ozonized oxygen would convert the "bivalent" anti-Rh "agglutinating" antibody to "univalent" "blocking" antibody. Boyd[1] failed to produce such a change by exposing agglutinating sera to photoflood lights. To study the effect that ozonized oxygen would exert in this direction, anti-Rh_o agglutinating serum, having an end titer of 1:64 against a 2% saline suspension of group O Rh_o erythrocytes, was treated and titrated in the same manner as were the serum specimens in our previous tests. The agglutinating antibody in this serum was completely destroyed by a 13-minute treatment with ozonized oxygen. After titration had shown that the Rh_o antibody had been destroyed, there was added to each dilution of serum and erythrocyte suspension one drop of similarly diluted untreated anti-Rh_o serum; the tube containing the 1:2 dilution of treated serum received an additional drop of 1:2 untreated serum, the 1:4 dilution received a drop of 1:4 serum and so on. Each tube now contained one drop of treated and diluted serum, one drop of 2% group O Rh_o erythrocyte suspension, and one drop of untreated serum of the same dilution as the treated serum. The contents of the tubes were mixed by shaking the rack containing the tubes and all were then again incubated in the 37°C water bath. The results of this test are shown in Table V.

It is clear from Table V that, although the agglutinating antibody was destroyed by treatment with ozone, there was nothing produced in it which inhibited the action of the untreated anti-Rh_o agglutinating serum when, subsequently, one drop of each dilution of the untreated serum was added to each tube and then incubated in the water bath at 37°C for an additional hour. There appears to be no conversion of anti-Rh_o agglutinating antibody to anti-Rh_o blocking antibody by treatment with ozone.

In order to evaluate the potential destructive effects of foaming on proteins and related isohemagglutinins, anti-Rh" serum was subjected to equivalent 10-minute flows of air, O_2, and ozonized O_2. The control agglutinating titer was 1:8. Following the bubbling of air the agglutinating titer was 1:4; for oxygen, 1:4; whereas the agglutinating titer for comparable exposure to ozonized oxygen was 1+ for the undiluted treated specimen. It will be noted in Table VI that some change was evident in the terminal titer. The intensity of agglutination for the lower dilutions showed no change with air and oxygen whereas a profound change was present with ozonized oxygen. Oxidation as effected by ozone in combination with oxygen is more potent than oxygen alone.

With the use of ozonized oxygen considerable macroscopic evidence of denaturation was evident. In most instances with prolonged exposure there developed a gummy, gelatinous precipitate which adhered to the glass rod and sides of the container. With short exposures macroscopic evidence of this precipitate was frequently absent.

Summary: It has been demonstrated that the blood group isoagglutinins, immune anti-A agglutinin, and Rh agglutinating and blocking antibodies are inactivated or destroyed relatively rapidly with 1:1000 dilution of ozone in oxygen.

So far as Rh agglutinating and blocking antibodies are concerned, our results are in essential agreement with the observations of others with respect to the action of physical factors such as temperature and pressure.

There appears to be no conversion of Rh_o agglutinating antibody to Rh_o blocking antibody by treatment with ozone.

TABLE V.

Effect of Ozone in Converting Anti-Rh₀ Agglutinating Antibody to Blocking Antibody.

Treatment of anti-Rh₀ agglutinating serum	Test erythrocytes 2% suspension in saline	Serum dilutions in saline								
		1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
Untreated	O Rh'	++	++	++	++	++	+	-	-	-
	O Rh₂	++	++	++	++	+	+	+	-	-
	O Rh₀	++	++	++	++	+	+	-	-	-
	O Rh-	-	-	-	-	-	-	-	-	-
Treated 13 min. with ozone	O Rh'	-	-	-	-	-	-	-	-	-
	O Rh₂	-	-	-	-	-	-	-	-	-
	O Rh₀	-	-	-	-	-	-	-	-	-
	O Rh-	-	-	-	-	-	-	-	-	-
1 drop treated serum plus 1 drop untreated	O Rh'	++	++	++	++	+	+	-	-	-
	O Rh₂	++	++	++	++	+	+	±	-	-
	O Rh₀	+	+	+	+	+	+	-	-	-
	O Rh-	-	-	-	-	-	-	-	-	-

+++, ++, +, ± = Varying degrees of agglutination of Group O Rh cells.
— = No agglutination.

TABLE VI.

Comparative Effects of Air, Oxygen, and Ozonized Oxygen on Anti-Rh'' Agglutinating Serum.

Treatment of anti-Rh'' agglutinating serum	Test erythrocytes 2% in saline	Serum dilutions in saline					
		Undil.	1:2	1:4	1:8	1:16	1:32
Untreated control	Rh'	+++	+++	++	++	+	-
	Rh''	+++	+++	++	+	-	-
10 minutes exposure to air	Rh₂	+++	+++	++	+	-	-
	Rh'	+++	+++	++	-	-	-
	Rh''	++	+	+	-	-	-
10 minutes exposure to oxygen	Rh₂	+++	++	+	-	-	-
	Rh'	++	++	+	-	-	-
	Rh''	++	+	-	-	-	-
10 minutes exposure to ozonized oxygen	Rh₀	+	+	-	-	-	-
	Rh'	-	-	-	-	-	-
	Rh''	±	-	-	-	-	-
	Rh₀	-	-	-	-	-	-

+++, ++, +, ± = Varying degrees of agglutination of Rh'' cells.
— = No agglutination.

parable to that used by Proger and Dekaneas in their studies.[5,6]

The acute altitude survival test has been described in detail elsewhere.[2,3] Exposure was carried out in a decompression chamber with sufficient air flow to prevent any perceptible increase in carbon dioxide content. The survival rates reported represent the percentage of animals alive at the conclusion of the test, since no delayed deaths were noted. All animals were exposed for one hour at the test altitude. Moreover, they were exposed simultaneously, thus eliminating any possible differences in survival rate due to variations in temperature and humidity within the chamber or such other unpredictable variables as handling and feeding.

Results and Discussion. The results of exposure with and without parenteral cytochrome C were as shown in Table I. A graphic presentation is given in the accompanying chart.

It is apparent that cytochrome C was not effective in protecting the intact rat against severe anoxia. If injected cytochrome C is capable of increasing the ability of tissues to utilize oxygen under conditions of anoxia, one might expect administration of this respiratory enzyme to increase the ability of the intact organism to withstand the anoxia of high altitude exposure. The present experiments give no indication as to why this expected action di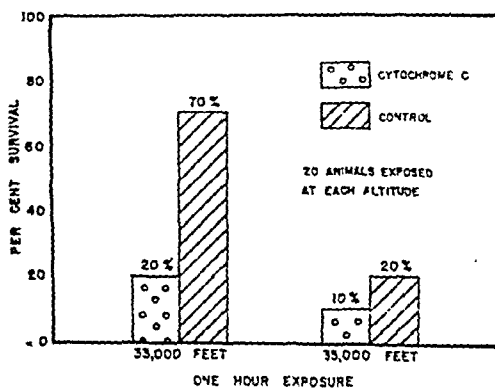d not occur. It is quite possible that the cytochrome C, being a protein whose molecular weight is at least 13,000, was unable to penetrate intact cells. However, there are indications that even had this occurred, it would not have affected the oxygen consumption of the cells.[9]

EFFECT OF INJECTED CYTOCHROME C ON ALTITUDE TOLERANCE OF NORMAL RATS

FIG. 1.

Summary. This report presents data concerning the ability of rats treated with cytochrome C to withstand the anoxia of high altitude exposure. The survival rates of experimental animals given an intraperitoneal injection of 5 mg of cytochrome C in isotonic saline were compared with those of control animals treated with a similar amount of saline. Groups of control and experimental animals were exposed simultaneously for periods of one hour. Altitudes of 33,000 and 35,000 feet were employed. In both instances, the survival rate was higher in the control group. In is concluded, therefore, that the intraperitoneal injection of cytochrome C is not effective in protecting rats against the anoxia of high altitude exposure.

TABLE I.
10 animals in each experiment.

Exposure altitude	Treatment	% survival
ft		
33,000	Normal saline—2 cc	70
"	Cytochrome C—5 mg	20
35,000	Normal saline—2 cc	20
"	Cytochrome C—5 mg	10

9 Shack, J., *J. Nat. Cancer Inst.*, 1943, 3, 389.

16091

Effect of Cytochrome C Therapy on Altitude Tolerance of Normal Rats.

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(Introduced by A. Baird Hastings.)

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Previous studies have indicated that the ability of normal rats to withstand severe anoxia can be improved by a variety of procedures. Animals rendered polycythemic by the administration of cobalt were capable of increased work performance under conditions of severe anoxia.[1] Administration of a concentrated extract of adrenal cortex in sesame oil was followed by a marked improvement in ability to withstand short exposures to ordinarily lethal altitudes.[2] Animals given a 2-hour conditioning exposure to pressures equivalent to 18,000 or 26,000 feet altitude daily for 7 or 30 days developed improvement in their ability to withstand exposure to ordinarily lethal altitudes.[3] In these later studies, the improvement was most marked after conditioning at the higher altitude, and was accompanied by both polycythemia and adrenal gland hypertrophy. However, the improvement could not be explained solely on the basis of either polycythemia or adrenal gland hypertrophy, since it occurred following conditioning at lower altitudes, which failed to influence organ weight or morphology, and there was a lack of correlation between hematocrit increase and the survival rate of the conditioned animals. It is apparent, therefore, that the mechanisms responsible for the increased altitude tolerance found in the above-noted experiments are not entirely clear. In view of this circumstance, it has been considered advisable to appraise the effect of parenterally administered cytochrome

C upon altitude tolerance. This was undertaken because of the recently demonstrated influence of adrenalectomy on cytochrome oxidase and cytochrome C concentration[1] and because of the beneficial influence of parenterally administered cytochrome C on patients suffering from inadequate oxygenation of their tissues.[5,6,7]

Methods. Young, male, white rats of approximately 200 g average weight were used in these studies. The animals were maintained in wire mesh cages on a standard diet of Purina dog chow. Access to food and water was unrestricted until 5 hours prior to exposure to anoxia; after this, water only was allowed. Five mg of cytochrome C in 2 cc of isotonic saline were administered to the experimental animals in a single intraperitoneal injection 45 minutes prior to altitude exposure.* The control animals were handled in the same manner as the treated group, except that an equal volume of physiological saline was substituted for the cytochrome C solution.

The cytochrome C was isolated according to the technique of Keilin and Hartree which yields a product containing .34% Fe.[8] The concentration of cytochrome in the injected material was determined by spectrophotometric analysis. The amount given is com-

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1 Dorrance, S. S., Thorn, G. W., Clinton, M., Jr., Edmonds, H. W., and Farber, S., *Am. J. Physiol.*, 1943, **139**, 399.

2 Thorn, G. W., Clinton, M., Jr., Davis, B. M., and Lewis, R. A., *Endocrinology*, 1945, **36**, 381.

3 Thorn, G. W., Clinton, M., Jr., Farber, S., and Edmonds, H. W., *Bull. Johns Hopkins Hosp.*, 1946, **79**, 59.

4 Tipton, S. R., *Endocrinology*, 1944, **34**, 181.

5 Proger, S., *Bull. N. E. Med. Center*, 1945, **7**, 1.

6 Proger, S., and Dekaneas, D., *Bull. N. E. Med. Center*, 1945, **7**, 149.

7 Proger, S., and Dekaneas, D., *Science*, 1946, **104**, 389.

* It has been reported by Proger and Dekaneas[7] that intravenous, intramuscular, and intraperitoneal injections of cytochrome C increase the blood and organ content of cytochrome C.
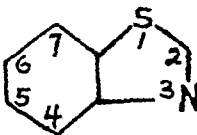
8 Keilin, D., and Hartree, E. F., *Biochem. J.*, 1945, **39**, 289.

TABLE I.
Tuberculostatic Action on Strains 607 and H37Rv.

No.	Compound	Formula	Mg % 607	H37Rv
1	Benzothiazole		10.	5.
2	2-Aminobenzothiazole	$C_7H_4NSNH_2$	>10.	
3	6- ,,	$C_7H_4NSNH_2$	0.25	0.1
4	6-Nitrobenzothiazole	$C_7H_4NSNO_2$	10.	
5	2-Mercaptobenzothiazole	C_7H_4NSSH	5.	
6	6-Amino,2-mercaptobenzothiazole	$H_2NC_7H_3NSSH$	5.	0.1
7	2-Methylbenzothiazole	$C_7H_4NSCH_3$	10.	
8	6-Amino,2-methylbenzothiazole	$H_2NC_7H_3NSCH_3$	0.1	0.01
9	6 Nitro,2- ,,	$O_2NC_7H_3NSCH_3$	10.	
10	2-Phenylbenzothiazole	$C_7H_4NSC_6H_5$	10.	
11	2-Chlorobenzothiazole	C_7H_4NSCl	5.	
12	6-Amino,2-ethoxybenzothiazole	$H_2NC_7H_3NSOC_2H_5$	10.	0.7
13	6-Nitro,2 ,,	$O_2NC_7H_3NSOC_2H_5$	>10.	
14	6-Amino,2-isopropoxybenzothiazole	$H_2NC_7H_3NSOCH(CH_3)_2$	2.5	0.35
15	6-Nitro,2- ,,	$O_2NC_7H_3NSOCH(CH_3)_2$	10.	
16	6-Amino,2-(n)butoxybenzothiazole	$H_2NC_7H_3NSOC_4H_9$	2.5	0.0025
17	6-Nitro,2- ,,	$O_2NC_7H_3NSOC_4H_9$	1.	
18	6-Amino,2-butoxyethoxybenzothiazole	$H_2NC_7H_3NSOC_2H_4OC_4H_9$	>10.	
19	6-Nitro,2- ,,	$O_2NC_7H_3NSOC_2H_4OC_4H_9$	5.	
20	6,2-Diaminobenzothiazole	$H_2NC_7H_3NSNH_2$	0.25	0.01
21	6-Nitro,2-aminobenzothiazole	$O_2NC_7H_3NSNH_2$	2.5	
22	6-Amino,2-hydroxybenzothiazole	$H_2NC_7H_3NSOH$	10.	
23	6-Nitro,2- ,,	$O_2NC_7H_3NSOH$	2.5	
24	6-Amino,2-chlorobenzothiazole	$H_2NC_7H_3NSCl$	0.025	0.0025
25	6-Nitro,2- ,,	$O_2NC_7H_3NSCl$	1.	
26	6'-Aminobenzothiazolyl,2'-(N)-morpholine	$H_2NC_7H_3NSNC_4H_8O$	>10.	
27	6'-Nitrobenzothiazolyl,2'-(N)-morpholine	$O_2NC_7H_3NSNC_4H_8O$	>10.	
30	5-Amino-2-isopropoxypyridine	$H_2NC_5H_2NOCH(CH_3)_2$	1.	0.1

The above compounds were prepared in this laboratory under the direction of Dr. F. A. French with the following exceptions: Nos. 1, 2, 5, 6, 7, 10 and 11 were obtained from the Eastman Kodak Co. (highest purity).

Control experiments were performed with propylene glycol alone in 1:50 dilution. No bacteriostatic effect was noted.

their bacteriostatic activity. Compounds Nos. 16 and 20 were tested against the following organisms: S. aureus, E. coli, Proteus vulgaris, Streptococcus pyogenes and Ps. pyocyaneus. The results were essentially negative.

The in vivo results are summarized in Table II. 6-amino-2-(n)butoxybenzothiazole and 6,2-diaminobenzothiazole were highly effective. These compounds were approximately twice as active as 4,4'diaminodiphenylsulfone (No. 28) and about ¾ as effective as streptomycin (No. 29). 6-Amino-2-isopropoxy and 6-amino-2-ethoxybenzothiazole (Nos. 14 and 12) were also significantly active therapeutically. Animals treated with

6,2-diaminobenzothiazole showed no enlargement of the thyroid gland. This is of interest because this compound contains a thioureoid structure similar to that found in 2-aminothiazole which has been reported to inhibit thyroid activity.[4]

Discussion. 6-Aminobenzothiazoles with 5 types of substituents in the 2- position have been found to be highly active against tubercle bacilli. The 2- substituents included alkoxy, alkyl, amino, chloro and mercapto. The invariant characteristic of the group is the possession of an aromatic amino group

4 Williams, R. H., Kay, G. A., and Solomon, B., Am. J. Med. Sci., 1947, 213, 198.

16092

Chemotherapy of Experimental Tuberculosis with Benzothiazole Derivatives.*

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In the following experiments a new class of tuberculostatic agents is described. A systematic evaluation of the bacteriostatic action of various benzothiazole derivatives on the growth of tubercle bacilli *in vitro* was attempted. Many of the compounds were also investigated *in vivo* for their therapeutic action on tuberculous animals. A few benzothiazole derivatives have previously been evaluated by Everitt and Sullivan[1] for their fungicidal action and as antivirus agents by Kramer and associates.[2] The authors[3] have reported the effect of several benzothiazole sulfones in experimental tuberculosis.

Methods. The bacteriostatic studies were initially performed on the avirulent strain of human tubercle bacilli, No. 607 of the American Type Culture Collection, using Lowenstein's liquid asparagin media, at a pH of 7.5. Many of the compounds were also tested on the virulent human strain, H37Rv, in Proskauer and Beck media. The chemicals were dissolved in propylene glycol to make 0.5% solutions. Subsequent dilutions were made directly into the aqueous media, without further heating. Each tube was inoculated with one loopful of fresh culture of tubercle bacilli. The avirulent cultures were read at the end of 3 days; the virulent cultures at 18 days. The highest bacteriostatic dilution was

taken as that dilution in which there was less than 50% of control growth.

Animal Experiments. Nine to 12 guinea pigs, averaging 400 g in weight, were used to evaluate each compound. The animals were inoculated in the left groin with 0.03 mg of H37Rv tubercle bacilli. The drugs were administered orally, suspended in water, by dropper once daily. Therapy was started on the day of infection. The dosage of the drugs was increased gradually for the first 2 weeks of the experiment. The dosage of all drugs approached the chronic maximum tolerated dose. The experiments were terminated at the end of 6 weeks. Pathology was evaluated numerically, based on a maximum of 4 for each organ (glands, spleen, liver and lungs); with a maximum of 16 for all organs. Hemoglobin and weight determinations were made at the end of the experiments.

Results. The *in vitro* bacteriostatic results are recorded in Table I. The benzothiazole ethers (Nos. 12 to 19 incl.) were the most extensively investigated group. The compound 6 - amino - 2 - (n)butoxybenzothiazole (No. 16) was bacteriostatic in extremely high dilution (1:40,000,000) on H37Rv. This activity was a thousand times as great as its bacteriostatic effect on the avirulent strain 607. All compounds tested were more bacteriostatic against the virulent culture. Five other types of benzothiazole derivatives were tuberculostatic in low concentrations: 6,2-diaminobenzothiazole (No. 20); 6-amino-2-chlorobenzothiazole (No. 24); 6-aminobenzothiazole (No. 3); 6-amino-2-mercaptobenzothiazole (No. 6) and 6-amino-2-methylbenzothiazole (No. 8). In general, the compounds were bacteriostatic rather than bactericidal. Compounds Nos. 16, 20 and 24 were tested in the presence of 20% defibrinated horse blood. They retained most of

1 Everitt, E. L., and Sullivan, M. X., *J. Wash. Acad. of Sci.*, 1940, **30**, 125.

2 Kramer, S. D., Greer, H. A., and Szobel, D. A., *J. Immunol.*, 1944, **49**, 273.

3 Freedlander, B. L., and French, F. A., *Am. Rev. Tuberc.*, in press.

symmetry of the ether group is very important. The chain length and symmetry of the ether group may very well maximize a tendency for proper molecular orientation and binding at some very important intracellular interface or enzyme locus. As evidence of the importance of symmetry the data of Friedman *et al.*[9] may be cited. With the 2-alkoxy-5-aminopyridines, using the n-amyl ether as unity the activities against strain No. 607 are: i-amyl -0.03, 3-n-amyl -0.5, n-butyl -1, i-butyl -.07, sec-butyl -0.016. These orientation factors may be much more important than the commonly known one of an optimal distribution coefficient, dependent on chain length, producing a maximal drug concentration in some lipoid phase.

The high order of specificity of some of the benzothiazole derivatives for strain H37Rv relative to strain No. 607 is encouraging. In the case of 6-amino-2-(n)-butoxybenzothiazole the activity ratio for the two strains is 1000. With other benzothiazole derivatives the ratio varies from 2 to 50. With the aryl-sulfones (extensively studied) the activity ratios were close to unity.

Those benzothiazole derivatives which were tested *in vivo* showed a low or moderate chronic toxicity. In the case of 6-amino-2-(n) butoxybenzothiazole a definite hypnotic effect appeared at the maximum tolerated dose. This is not unexpected for an ether-amine nor for a compound which may be regarded as a cyclic thiocarbamic ester. Hemotoxic effects which are so prominent with derivatives of diaminodiphenylsulfone were absent with the benzothiazole compounds. For comparison of toxicity and activity, 2-isopropoxy-5-aminopyridine (No. 30) (somewhat comparable to 6-amino-2-isopropoxybenzothiazole of this series) was evaluated *in vivo*. While this compound is not at the pyridine series optimum the comparison to equivalent benzothiazole compounds is significant. It may be noted that much smaller dosages of the pyridine ether were tolerated, marked liver damage occurred, and no significant effect on the infection was noticed.

Many types of compounds possessing active aromatic amino groups are tuberculostatic. Feinstone, Friedman and co-workers found many amino pyridines, pyrimidines and benzenes to be active. The authors[12] have noted fair activities with some amino acridines and phenothiazines. It may be suspected that some tuberculostatic activity is possessed by a great variety of amino-cyclic compounds in which the amino group is aromatic in character and is suitably activated.

Further studies of benzothiazole derivatives, including solublized derivatives of 6-amino-2-(n)butoxybenzothiazole, are being undertaken.

Summary. 1. A new class of highly tuberculostatic agents of low toxicity is described. This group comprises many 2,6- substituted derivatives of benzothiazole. 2. Several of these derivatives are active therapeutic agents in guinea pig tuberculosis. 3. Structure activity relations and possible modes of action are discussed.

12 French, F. A., and Freedlander, B. L., unpublished data.

TABLE II.
Summary of *in vivo* Experimental Data.

No.		Avg daily dosage mg per animal	*Path. ratio, Controls ——— Treated	Relative wt gain, g deviation from controls	Hglb. % deviation from controls
12	6-Amino-2-ethoxybenzothiazole	50-150	2.40	—87.	+14.
13	6-Nitro-2- ,,	50-150	1.60	—72.	0.
14	6-Amino-2-isopropoxybenzothiazole	50-150	2.28	—58.	+14.
16	6-Amino-2-(n)butoxybenzothiazole	50-100	3.05	—67.	— 3.
18	6-Amino-2-butoxyethoxybenzothiazole	75-125	1.75	—80.	0.
19	6-Nitro-2- ,,	50-130	1.04	+25.	0.
20	6,2-Diaminobenzothiazole	50-150	3.03	—68.	0.
24	6-Amino-2-chlorobenzothiazole	50-100	1.59	—50.	— 8.
28	4,4'-Diaminodiphenylsulfone†	50-100	1.66	—32.	—20.
29	Streptomycin†	20 (hypo)	3.9	— 5.	— 5.
30	5-Amino-2-isopropoxypyridine	30	1.14	—137	— 9.

* "Pathological Ratio" is the result of the average numerical pathological rating of the control animals divided by that of the treated animals.

† Diaminodiphenylsulfone and streptomycin are included for comparison.

in the 6- position and of an activating substituent in the 2- position. Kumler and Daniels[5,6] proposed a theory of activity of sulfonamides and aminoacridines in which the reactive character of the aromatic amine group is correlated with bacteriostatic activity. The aromatic amino group must be present, of course, in a molecule of suitable overall structure. This theory may be applied to these new tuberculostatic agents. The 6-amino group is an aromatic amino group and the 2- substituents may be regarded as activating groups. Using 6-aminobenzothiazole, where the 2- position is occupied by hydrogen, as a base level, one may calculate activity ratios with strain H37Rv as the test organism. With the following 2-substituents the activity ratios are: CH_3 -10, SH -1, OC_2H_5 -0.14, NH_2 -10, $OC_4H_9(n)$ -40. In a previous paper[3] it was shown that a 6-aminobenzothiazole substituted with 2-arylsulfonyl groups, which are electroacceptors, had only a low order of activity. The present compounds with electrodonor groups in the 2- position are as much as 1,000 times as active. Unfortunately, as yet, no adequate quantitative measure of the reactive character of the aromatic amine group is available. Qualitative comparisons can be made

if adequate allowance is made for other important factors affecting drug activity.

In the case of the 6-amino-2-alkoxybenzothiazoles it is apparent that the distribution coefficient is of considerable significance and this effect is much more noticeable with H37Rv than with strain No. 607. 6-Amino-2-ethoxy, isopropoxy, and (n)butoxybenzothiazoles all have about the same activity against strain No. 607 but the butyl ether is 300 times as active as the ethyl ether against strain H37Rv. The generic similarity of these benzothiazole ethers to the pyridine and other ether-amines studied by Feinstone, Friedman and co-workers[7-11] suggests that certain generalizations of some validity concerning both groups of compounds can be made. They found an optimum activity with ether chains of 4 to 6 carbon atoms. In addition it is noticeable from their data that the

5 Kumler, W. D., and Daniels, T. C., J. Am. Chem. Soc., 1943, 65, 2190.

6 Daniels, T. C., personal communications.

7 Friedman, H. L., Braitberg, L. D., Tolstoouhov, A. V., and Tisza, E. T., J. Am. Chem. Soc., 1947, 69, 1795.

8 Braitberg, L. D., Robin, M., Friedman, H. L., and Tisza, E. T., J. Am. Chem. Soc., 1947, 69, 2005.

9 Friedman, H. L., Braitberg, L. D., Tolstoouhov, A. V., and Tisza, E. T., J. Am. Chem. Soc., 1947, 69, 1204.

10 Feinstone, W. H., Friedman, H. L., Rothlauf, M. V., Kelly, A. M., and Williams, R. D., J. Pharm. and Exp. Therap., 1947, 89, 153.

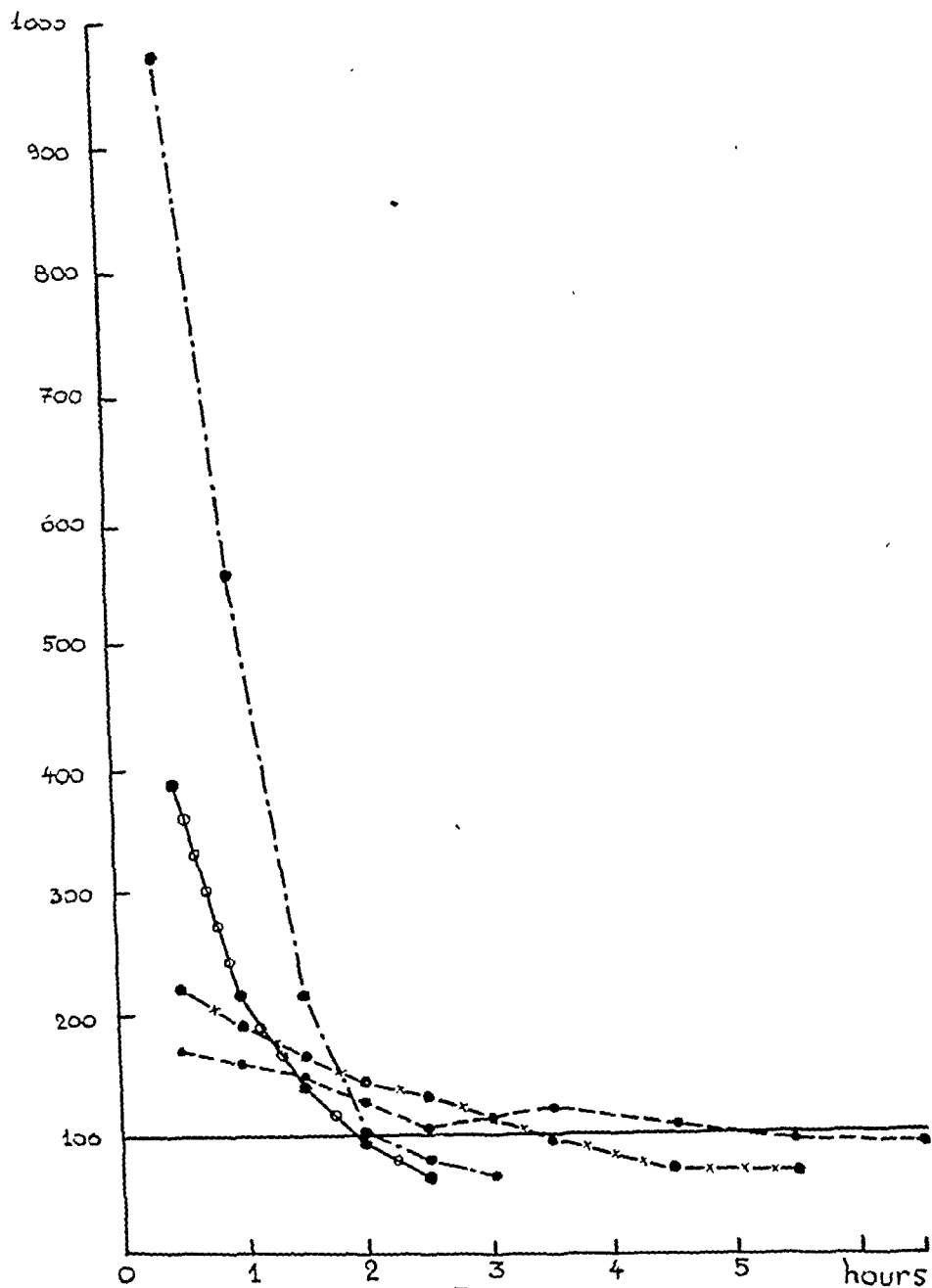11 Feinstone, W. H., PROC. SOC. EXP. BIOL. AND MED., 1946, 63, 153.

FIG. 1.

Effect of X-radiation on the respiration of nuclei of fowl erythrocytes and of whole erythrocytes. The data given are the O_2 consumption in the experimental vessels, expressed as a percentage of the O_2 consumption observed in the control vessels at a given time.

——————— Non-irradiated nuclei or erythrocytes.
O—O—O Nuclei irradiated with 500,000 r.
—·—·—· Nuclei irradiated with 1,000,000 r.
— — — — Erythrocytes irradiated with 500,000 r.
——X—— Erythrocytes irradiated with 1,000,000 r.

Influence of X-rays on Respiration of Nuclei of Fowl Erythrocytes.*

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In recent years a series of publications have appeared dealing with the influence of X-rays on respiration. Williams and Scheard[1] demonstrated increased oxidation in irradiated frog's skin. Lacassagne[2] working with yeasts also observed increased O_2 consumption after irradiation.

In a previous paper[3] we have demonstrated that fowl-erythrocytes irradiated with X-rays consume more oxygen than do unirradiated red cells. The purpose of the present study was to investigate the respiration of the irradiated nuclei of fowl erythrocytes and to compare the results with those obtained with whole erythrocytes.

Methods. (a) Preparation of nuclei. Nuclei were prepared according to the technique of Dounce and Lan. White Leghorn hens were bled by cardiac puncture and 20 cc of blood were sterilely withdrawn into 2 ml of 3% solution of Na citrate. The erythrocytes were centrifuged twice washed in 0.9% saline and the sedimented cells were resuspended in saline to the original volume of the blood. In order to induce hemolysis a solution of 0.15 g of saponin in 5 ml of an 0.11 M solution of phosphate (pH 7) was added to each 100 ml of erythrocyte suspension. After hemolysis was completed (within 2-3 minutes), the hemolysate was centrifuged and subsequently washed 5 or 6 times by centrifuging in 0.9% saline and was finally resuspended in this solution up to a concentration of approximately 10-18 mill/mm³. The nuclei

were then counted in a counting chamber.

(b) Irradiation. The X-ray tube used in these experiments was operated at a tension of 35 KV at a current of 15 mA with a copper anticathode. The window was aluminum foil 30 μ in thickness. Absorption analysis showed that the rays which penetrated through the window were mainly copper X-rays. The distance between the vessel in which the nuclei were irradiated and the focus was 50 mm and the X-ray intensity was about 60,000 r/min. Irradiation was performed on 0.5 ml portions of the nuclei suspensions in flat-bottomed, shallow glass dishes of 15 mm diameter. The suspension layer did not exceed 3 mm in thickness. Irradiation doses of 0.5 Mil., 1 Mil., and of 1.5 Mil. were applied.

(c) Measurement of respiration. Respiration was measured in a Warburg *apparatus* at 39-40°. Two ml of suspension were used per vessel for each estimation. Five to six 0.5 ml portions had, therefore, to be irradiated consecutively and combined. Control estimations of non-irradiated nuclei from the same sample of suspended nuclei were carried out in every case. The samples to be irradiated, as well as those already irradiated and the controls were kept under identical conditions of temperature throughout. The first reading was made 20 minutes after inserting the manometers in the water-bath (about half an hour after the termination of irradiation). Readings were taken initially at intervals of 30 minutes and later at intervals of 60 minutes.

Results and Discussion. As Dounce has already shown chicken erythrocyte nuclei continue for some days to exhibit a weak but demonstrable respiration. In our study this respiration of isolated nuclei was ⅛ to 1/10 of that of whole erythrocytes. Absolute values varied with individual chickens. X-rays had

* Aided by a grant of the British Empire Cancer Campaign.

1 Williams, M. M. D., and Scheard, C., *Protoplasma*, 1932, 15, 396.

2 Lacassagne, Schoen et Beraud, *Ann. des Fermentations*, 1939, 5, 124.

3 Frankenthal, L., and Back, A., *Biochem. J.*, 1944, 38, 351.

16094

Relationship of Weight, Venous Pressure and Radiosodium (Na^{22}) Excretion in Chronic Congestive Heart Failure.[*]

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New Orleans.*

During the course of tracer studies with Na^{22}[†] in 2 patients with chronic congestive heart failure and one normal subject, the relationship of weight, venous pressure and sodium excretion was observed.

Each subject received an intravenous injection of a tracer dose of Na^{22} (activity of 12,500,000 counts per minute[1]). Over a 2-month period, all urine samples were collected separately and the concentration of radiosodium determined for each, as well as for daily samples of blood serum. All counts were corrected so as to correspond to an initial injection of Na^{22} with an activity of 20,000,-000 counts per minute per 100 lb body weight. Weights and venous pressures[2] were recorded daily.

Results. (1) *Behavior of weight, venous pressure and sodium excretion with the subjects on a low sodium diet.* In all 3 subjects during a 22-day period of low sodium intake (<1.7 g NaCl/day), the weight and venous pressure varied concordantly, although the daily changes were not always concordant (Fig. 1, 2, and 3). The associated changes in sodium excretion were inversely related to those of weight and venous pressure. The patient whose weight and venous pressure were decreasing excreted the largest per cent of the injected Na^{22} during this period, while the

least was excreted by the patient whose weight and venous pressure were increasing.

(2) *Effect of adding sodium chloride to the diet.* Twelve grams of NaCl per day were added to the diet of the normal subject and the patient with congestive heart failure who was recovering (Patient No. 1). This resulted in a marked increase in the sodium clearance and in the sodium excretion of the normal subject, but there was only a slight increase in the clearance without an increase in the total Na^{22} excretion in the patient with heart failure. There was a slight increase in the weight and venous pressure associated with the decrease in total Na^{22} output in the patient with congestive heart failure.

(3) *Effect of Mercuhydrin (mercurial diuretic).* The most pronounced effects of Mercuhydrin (the sodium salt of methoxyoxi-mercuripropylsuccinylurea with theophylline)[‡] were an increase in sodium clearance and a decrease in weight in all subjects. Other effects included an increased excretion of water and sodium, usually with a proportionately greater increase in sodium excretion than in water output. Often there was a decrease in venous pressure. It may be noted that frequently during the periods following the diureses due to Mercuhydrin, the excretion of sodium and water was less than had occurred during and just prior to the diureses.

(4) *Relation of sodium excretion to fluid output.* In general, the day to day fluctuations in the excretion of water and of sodium were concordant, the latter fluctuating more, particularly following Mercuhydrin.

(5) *General trends.* During the 60-day period of observation both the venous pressure and weight remained within a constant range

[1] Reaser, Paul B., and Burch, George E., PROC. SOC. EXP. BIOL. AND MED., 1946, 63, 543.

[2] Burch, G., and Winsor, T., *J. A. M. A.*, 1943, 123, 91.

TABLE I.

Influence of Irradiation on O_2 Consumption of Nuclei of Fowl Erythrocytes.

μl O_2 Consumed by 2 ml of Nuclei Suspension 10 Mill Nuclei/1 mm³ After 30 to 180 min.

Irradiation dose (in millions r)	30 min.	60 min.	90 min.	120 min.	150 min.	180 min.
0.5	22.9	41.5	52.1	59.1	64.4	
0	5.9	14.4	22.0	29.6	38.1	
1	68.8	101.2	120.5	131.2	140.7	148.9
0	7.1	14.7	23.6	34.8	47.1	59.6
1.5	16.7	28.6	35.9	41.2	46.6	50.5
0	2.0	4.0	8.0	12.6	17.8	23.9

an accelerating effect on the respiration of nuclei and this effect was far more pronounced than that on whole erythrocytes as may be seen in Fig. 1. Thus, the effect of ½ million r on isolated nuclei is about twice as great and the effect of one million r is more than 4 times as great as the corresponding effects on whole erythrocytes.

In Fig. 1 we give the rate of O_2 consumption of normal and of irradiated nuclei and of whole erythrocytes irradiated and non-irradiated. The rate of respiration of non-irradiated erythrocytes and that of non-irradiated nuclei was arbitrarily designated as 100 (the horizontal base line). Values for irradiated nuclei observed at the same times were computed relatively to this normal value. The curves thus represent percentage increase or decrease in the respiration of irradiated nuclei compared with non-irradiated controls, plotted against time. The same figure also presents the curves describing the intensified respiration of whole erythrocytes irradiated also with identical doses of ½ and one million r. The fact that the curves for nuclei for both doses fall sharply and that in both cases the normal is reached simultaneously is striking.

Table I presents a series of typical experiments with different doses of X-rays. These figures show that after exposure to any of the given doses the intensity of respiration of the irradiated nuclei is initially high, becomes gradually weaker and falls to below normal after 3 hours. A similar, though somewhat delayed course is taken by the respiration of whole erythrocytes after irradiation, in which case the process of inhibition sets

in much later.

It is still impossible to explain the process of X-ray activation described by various authors. The observed difference between the respirations of irradiated erythrocytes and irradiated nuclei alone, however, may be due to the protective effect of cytoplasmic proteins against the influence of X-rays. In order to verify this assumption we added 0.5% albumin or hemolysate to the nuclei before irradiation, thereby considerably weakening the stimulation.

Dale,[4] Friedewald,[5] Evans[6] and others have described such a protein effect on viruses, enzymes and sea-urchin sperm. It may be assumed that the proteins present in erythrocytes (globulin, hemoglobin, etc.) also exert a similar protective effect on the influence of X-rays on the respiration of whole erythrocytes.

Summary. 1. The effect of X-ray irradiation on the respiration of the nuclei of fowl erythrocytes has been investigated. 2. The respiration of these nuclei was temporarily stimulated by treatment with doses of 500,000 to 1,500,000 r. This stimulation of nuclear respiration was more pronounced than that obtained with whole erythrocytes. 3. The addition of hemolyzed blood or albumin to the suspension of nuclei before irradiation has a protective effect against the activating influence of X-rays.

[4] Dale, W. M., Meredith, W. J., and Tweedie, M. C. K., *Nature*, 1943, **151**, 280.

[5] Friedewald, W. F., and Anderson, R. S., *J. Exp. Med.*, 1941, **74**, 463.

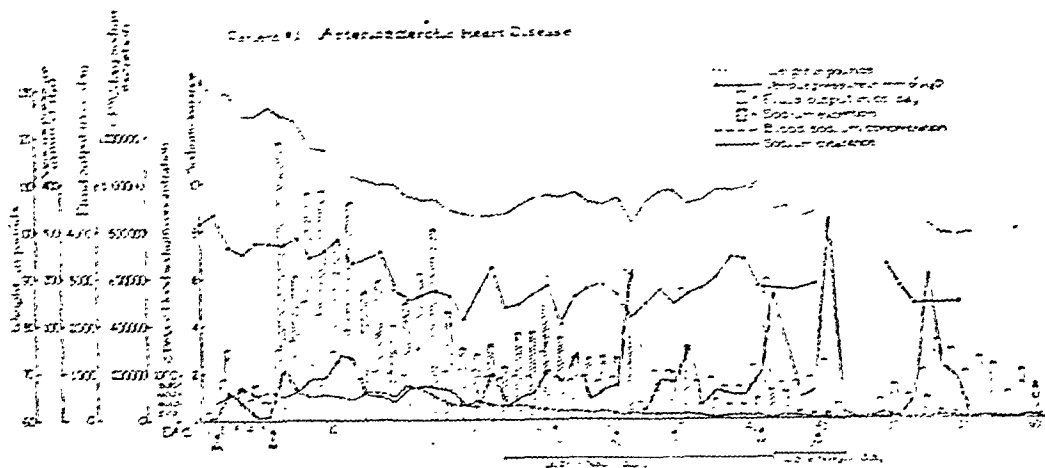[6] Evans, T. C., Slaughter, J. C., Little, E. P., and Failla, G., *Radiology*, 1942, **39**, 663.

FIG. 2.

Variations in weight, venous pressure, fluid output, radiosodium (Na^{22}) excretion, blood radiosodium concentration and radiosodium clearance in a 62-year-old negro male with senile arteriosclerotic heart disease in congestive heart failure (Functional Class IV) who improved under treatment. Weight and venous pressure decreased concordantly associated with an excretion of radiosodium exceeding that of the normal subject. Addition of 12 g of NaCl per day to the diet caused a concordant increase in weight and venous pressure concurrent with a decreasing excretion of radiosodium. Intramuscular injections of Mercuhydrin (M) in 2 cc doses caused increases in radiosodium clearance, total sodium excretion and total water output. One-half of the injected radiosodium was excreted in 25 days and 64% by the end of the study (60 days). The patient was on a low sodium diet except from the 22nd to 41st day. On the 15th day K^{42} was administered for other study purposes.
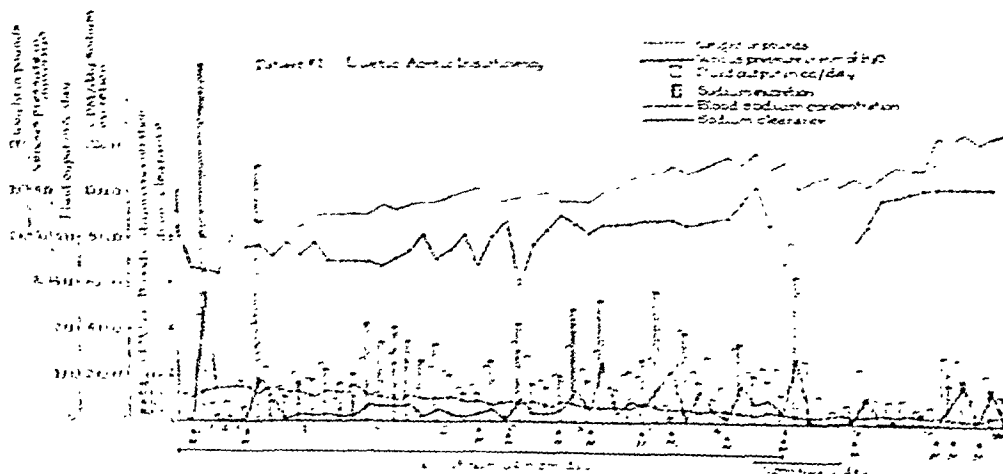

FIG. 3.

Variations in weight, venous pressure, water output, blood radiosodium (Na^{22}) concentration, radiosodium clearance and radiosodium excretion in a 46-year-old negro male with luetic aortic insufficiency admitted in congestive heart failure who decompensated further while under observation. The weight and venous pressure increased concordantly. The rate of excretion of radiosodium was less than that of the normal subject; only 42% of the injected Na^{22} was excreted in the 60 days. Intramuscular injections of 2 cc of Mercuhydrin (M) produced an increase in radiosodium excretion, blood radiosodium clearance and water output. The patient was on a low sodium diet during the entire study and digitalized during the first 45 days. On the 15th day, K^{42} was administered for other purposes.
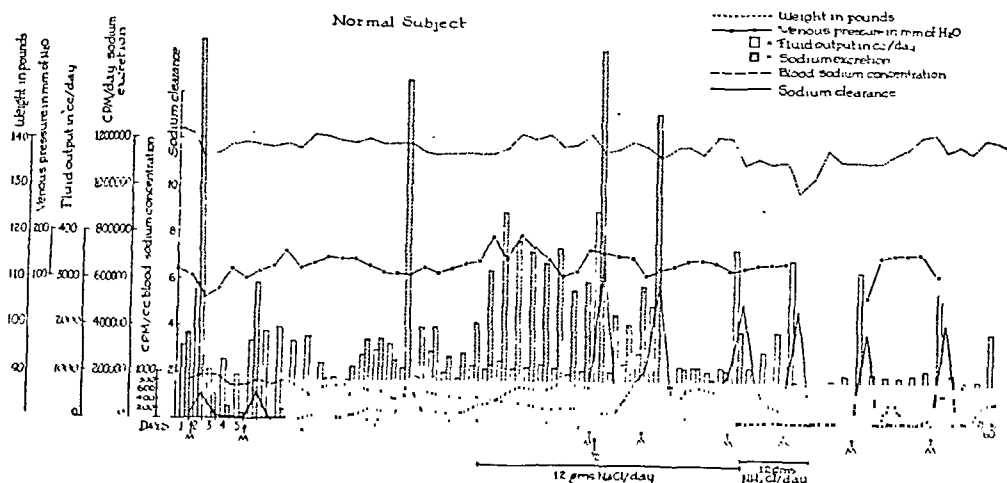
Fig. 1.

Variations in weight, venous pressure, fluid output, radiosodium (Na22) excretion, blood radiosodium concentration and blood radiosodium clearance in a normal 37-year-old negro male. The weight and venous pressure showed daily variations within a narrow range. The addition of 12 g of NaCl per day to the diet resulted in an increase in blood radiosodium clearance and total radiosodium excretion. Intramuscular injections (2 cc) of Mercuhydrin (M) resulted in increases in radiosodium clearance, fluid output and radiosodium excretion. One-half the injected radioactive sodium was excreted in 30 days and 69% was excreted by the end of the study (60 days). The subject was on a low sodium diet except from the 22nd to the 41st day. On the fifteenth day K42 was administered for other study purposes.

for the normal subject (Fig. 1), decreased in the patient recovering from failure (Fig. 2) and increased in the patient decompensating (Fig. 3).

The normal subject excreted 50% of the injected Na22 in the urine in 30 days and 69% by the end of 60 days (Fig. 4). The patient rapidly improving had excreted 50% in 25 days and 64% by the end of 60 days, while the patient decompensating had excreted only 42% at the end of the 60 days.

For a period of 5 hours following one of the injections of Mercuhydrin (on the 35th day) several serial determinations of weight and venous pressure were made. The behavior of weight, venous pressure, water output, Na22 excretion and Na22 clearance were similar for the briefer period to that described for the longer intervals. That is, weight and venous pressure were concordant and were inversely related to fluid and Na22 excretion and clearance.

Comments. The findings in this study were in accord with the generally accepted ideas of congestive heart failure, that is, the pa-

tients with congestive heart failure were found to excrete less than normal amounts of Na and water during the period that the failure was advancing, but during the period of improvement the excretion of salt and water exceeded that observed in the normal subject. The patient recovering from congestive heart failure excreted more sodium when on a low sodium diet than when on a high sodium one, while the reverse was true for the normal subject (Fig. 1, 2). Mercurial diuretics, digitalis and ammonium chloride were among the other agents, the influences of which were observed during the 60 days of continuous study.

While it was shown that changes in weight and venous pressure were concordant, but both varied inversely with the sodium and water excretion, a change in weight or venous pressure or sodium excretion could not be shown to precede a change in either of the other. Thus, from the data obtained, it is quite unlikely that a mere increase in intracapillary hydrostatic pressure is responsible for these changes. It could not be demonstrated that either sodium retention or in-

16095

Stratification of the Erythrocytes of Man by Ultracentrifuging.*

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The shape and structure of the mammalian erythrocytes have attracted the attention of many investigators.[1-4] The chief problems involved seem to center around a possible explanation for the bioconcave form assumed by the erythrocytes when floating freely in the plasma, and on efforts to deduce an architecture suitable to account for the function and physiological behavior of these cells.

Beams and Hines[5] found that it was possible to stratify the rat erythrocytes by ultracentrifugation. Similar studies dealing with the physical properties of the erythrocytes of man have been made and are herewith reported.

Material and Methods. Freshly drawn blood from the finger of an adult man was placed in the rotor of the ultracentrifuge which was subsequently sealed. After standing until the blood had clotted the centrifuge was started and operated at a pressure which delivered a centrifugal force of approximately 400,000 times gravity. After centrifuging for 20 to 30 minutes the blood was fixed in Champy's fluid for 12 hours. Paraffin sections were made of the clot which contained many erythrocytes in its meshes. These sections were bleached in potassium permanganate and oxalic acid and subsequently stained in Heidenhain's hematoxylin or Mallory's triple stain. Several other staining procedures were also tried, including supravital methods.

Results. The cells in Fig. 1 and 2 have been stained in Heidenhain's hematoxylin. They are slightly elongated and their typical biconcave appearance seems to have been altered. The centrifugal halves of the cells stain sharply with the hematoxylin while the centripetal portion remains unstained. It is evident that the erythrocytes possess a membrane which is capable of resisting considerable mechanical distortion. Fig. 3 and 4 show erythrocytes in profile that have been stained in Heidenhain's hematoxylin. The hematoxylin-stained material has been concentrated at the centrifugal end of the cells without greatly distorting their biconcave appearance. Because these cells were free to shift their positions relative to the centrifugal field during the process of removal from the centrifuge, they are not all oriented in the same direction in the photographs.

In Fig. 5 the cells were stained in Heidenhain's hematoxylin and acid fuchsin. In the isolated cells of the lower half of Fig. 5 separate layers may be observed: a hematoxylin-staining layer at the centrifugal end, a nonstaining area in the middle, and an acid fuchsin staining portion at the centripetal end. Similarly, 3 distinct layers may be observed following staining in Mallory's triple stain (Fig. 6). Here the centrifugal portion of the cells is stained orange, the middle red, and the centripetal end a light blue. The fact that they are more or less selectively stained by different dyes indicates that they are of a different chemical composition.

Discussion. The erythrocytes seem to contain at least three distinct substances which differ in their relative specific gravities and staining reactions. However, the chemical composition of these substances is at present unknown. Supravital preparations did not reveal clear evidence of stratification, although it was previously observed for the erythrocytes of the rat that the hemoglobin in some

1 Winthrobe, M. W., *Medicine*, 1930, 9, 195.

2 Krumbhaar, E. B., *Special Cytology*, Paul B. Hoeber, New York, 1932, 2, 555.

3 Ponder, E., *Protoplasma Monographien*, Berlin, 1934, 6, 66.

4 Isaacs, R., *Handbook of Hematology*, Paul B. Hoeber, 1938, 1, 5.

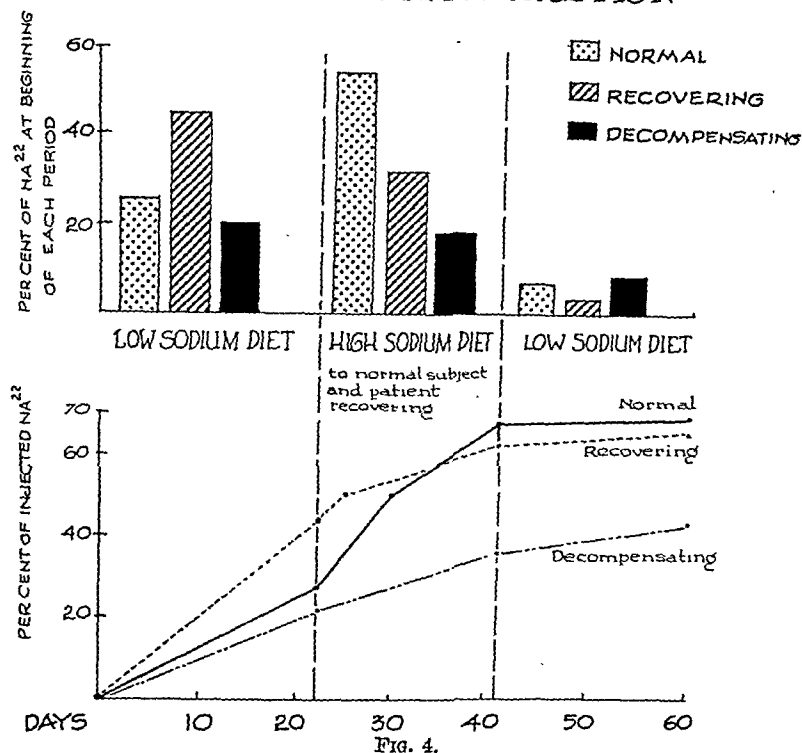5 Beams, H. W., and Hines, E. B., *Anat. Rec.*, 1944, 90, 155.

FIG. 4.

The rate of excretion of radiosodium (Na^{22}) as per cent of the total injected dose for the subjects studied. The study may be divided into 3 periods, (1) during the first 22 days the subjects were on a low sodium diet, (2) during the next 19 days the normal subject and the patient recovering from heart failure were receiving 12 g of NaCl per day, and (3) during the remaining period of the observation all 3 subjects were on a low sodium intake. The histograms show the relation of radiosodium output in the normal subject to that of the patients with heart disease, both while on a low sodium diet and while receiving 12 g of NaCl per day. These latter per cent values represent proportions of the amount of Na^{22} in the body at the onset of each of the 3 periods.

crease in venous pressure was the primary factor in edema formation or that edema predisposed to either of these factors. The actual mechanisms of the retention of Na and water in congestive heart failure remain unknown.

in these studies, although we have observed it in normal uncentrifuged erythrocytes treated by other methods.[6]

Summary. Three distinct substances which differ in their relative specific gravities have

been stratified by high centrifugal force in the erythrocytes of man. These substances seem to differ chemically for they may be differentially stained by certain dyes. The stretching of certain of the erythrocytes by centrifugal force indicates that they possess a membrane of considerable elasticity.

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[6] Jordan, H. E., Kindred, J. E., and Beams, H. W., *Anat. Rec.*, 1930, 46, 139.

16096

Influence of Epinephrine on Vasoconstrictor Action of Kidney Extracts.*

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Perfusion of rabbit's ears with saline extracts of minced kidney is usually associated with vasoconstriction. Minute amounts of histamine or histamine-like compounds present in the minced organ are thought to be responsible for this effect. When these are removed by dialysis, acetone-ether treatment and other methods involved in fractionation of the crude preparation, the vasoconstrictive action as determined with the rabbit's ear or the dog's tail, is lost.

It is reported, further, that the vasoconstrictive action of some kidney fractions as demonstrated by intravenous injection in the living animal may be absent when tested by the perfusion method.[1,2,3] Important conclusions, it will be recalled, have been drawn from this discrepancy as determined by the two methods of testing the same extract.[2,3] Little if any attention on the other hand has been given the reports of Schaumann,[4] Burn and Tainter[5] and more recently of Morton and Tainter.[6] These authors observed that various

sympathomimetic amines are vaso-inactive when perfused through isolated peripheral blood vessels. When, however, subthreshold amounts of epinephrine are added to the perfusate, strong vasoconstriction follows.

In the light of these findings the vasoactivity of various kidney extracts has been re-examined.

As test object the vessels of the isolated rabbit's ear were selected. This is a technique devised by Pisemski and Krawkow,[7] modified by Schlossman,[8] Kahlson and von Werz,[9] and further by Brandt and Katz.[10] More recently it has been extensively employed by Page[11] who incorporated pulsating pressure. It is the general opinion that this method adequately permits measurement of vasoconstrictive substances in perfusion fluids.

Materials and Methods. In general, the experiments followed the technique described by Landis[12] and by Morton and Tainter.[6] A constant, non-pulsating perfusing pressure

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[1] Hessel, G., *Klin. Wchnschr.*, 1938, 17, 843.

[2] Braun-Menendez et al., *Renal Hypertension*, translated by Dexter, L., Chas. C. Thomas, Springfield, Ill., 1946.

[3] Page, T. H., and Helmer, P. M., *J. Exp. Med.*, 1940, 71, 29, 495.

[4] Schaumann, O., *Arch. Path. Pharmacol.*, 1928, 138, 208.

[5] Burn, J. H., and Tainter, M. L., *J. Physiol.*, 1931, 71, 169.

[6] Morton, M. C., and Tainter, M. L., *J. Physiol.*, 1940, 98, 263.

[7] Krawkow, N. P. et al., *Z. f. die ges Exp. Med.*, 1922, 27, 127.

[8] Schlossman, E., *Arch. f. Exp. Path. u. Pharmacol.*, 1927, 121, 160.

[9] Kahlson, G., and von Werz, R., *ibid.*, 1930, 148, 173.

[10] Brandt, G., and Katz, G., *Z. f. Klin. Med.*, 1933, 123, 23.

[11] Page, T. H., *Am. Heart J.*, 1942, 23, 336.
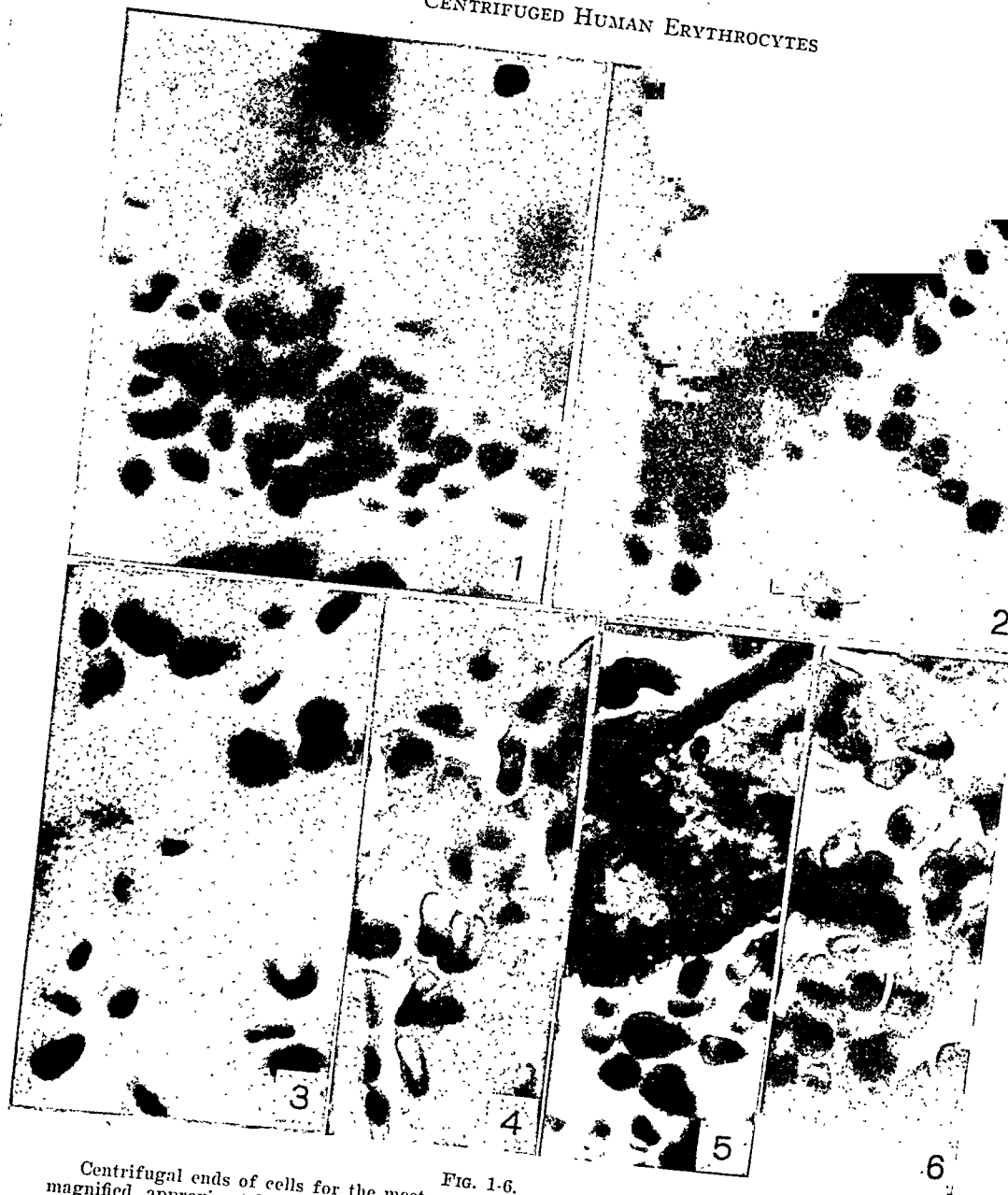
[12] Landis, E. M., et al., *Am. J. Physiol.*, 1943, 139, 26.

FIG. 1-6.

Centrifugal ends of cells for the most part directed toward the bottom of plate. All cells magnified approximately 1250 ×. 1 and 2. Stratified erythrocytes, hematoxylin stained. 3 and 4. Similarly treated cells in profile, normal shape evident. 5. Hematoxylin and acid fuchsin stained, 3 layers present. 6. Mallory's stained, three layers evident.

of the cells was displaced. The absence of a sharply visible stratification in the living unstained erythrocytes is not surprising because no internal structure can be seen in the normal cells with direct illumination, dark field. or with ultraviolet light.[3] Although the appearance of the stratified layers may be somewhat altered by different staining procedures. it is not likely that they represent artifacts any more than do stained structures of other cells treated in a similar way. No evidence of a stained reticulum was observed
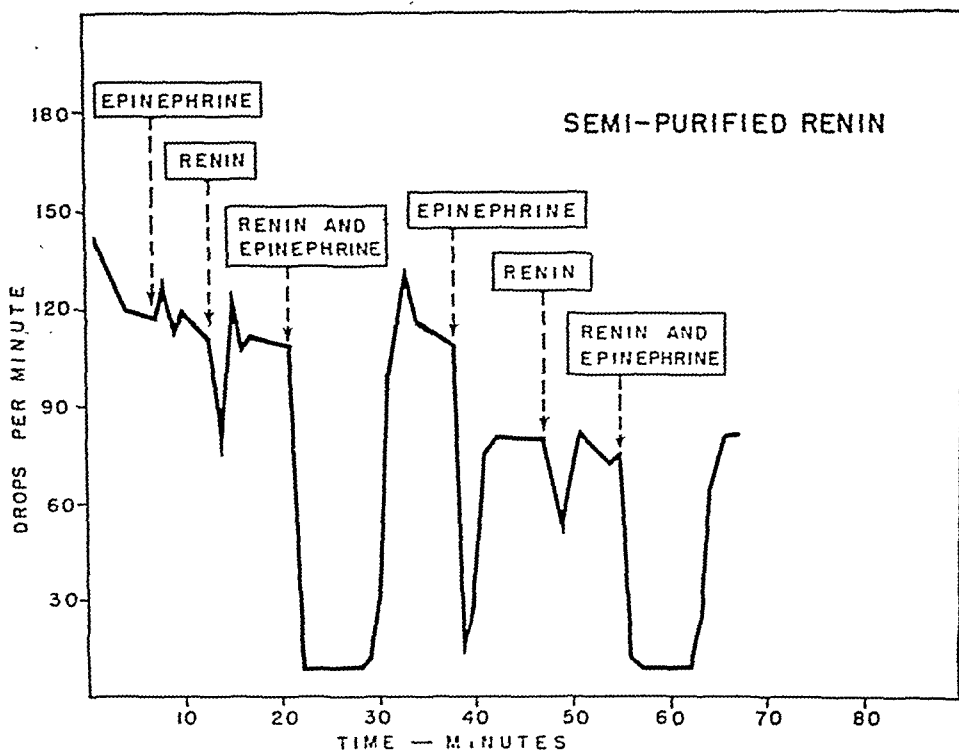
FIG. 2.

Vasoconstrictor action of a combination of semi-purified renin and epinephrine. Epinephrine amount used: 1 cc of 1:2,500,000.

resulting lyophilized protein mixture injected intravenously raised the blood pressure of a 10 kilo dog 30-40 mm of Hg.

Results. The saline extract of the dry powder produced no significant effect on the flow of the perfusate through the rabbit's ear in 10 trials. When combined with a quantity of epinephrine, in itself without effect on the perfused vessels, marked vasoconstriction followed. A typical experiment is recorded in Fig. 1.

A short constrictor effect was occasionally encountered on the second or third injection of amounts of epinephrine that were "subthreshold" on the first injection. This contrasted with the prolonged constriction that followed the combination of epinephrine and renin.

The second preparation gave equivocal results. In itself inactive on perfusion, vasoconstriction followed with addition of a subthreshold quantity of epinephrine in 16 of the 26 trials. The preparation was found to be only relatively soluble, as might have been anticipated after years of storage and this is thought to be associated with the inconstant results secured.

The findings were uniform with the recently prepared and quite soluble third preparation. No vasoconstriction followed when it was added to the isolated rabbit ear perfusate in many trials. When, however, it was supplemented with the above described minimal amounts of epinephrine marked vasoconstriction invariably resulted. This is illustrated in Fig. 2.

Summary. Protein fractions of hog kidney, in themselves inactive on perfusion, become strongly vasoconstrictive when supplemented with traces of epinephrine. While a similar influence of minute quantities of epinephrine on vasoconstriction is well known to occur with some sympathomimetic amines, insufficient information is at hand to clarify the pharmacological nature of the observed synergistic action.
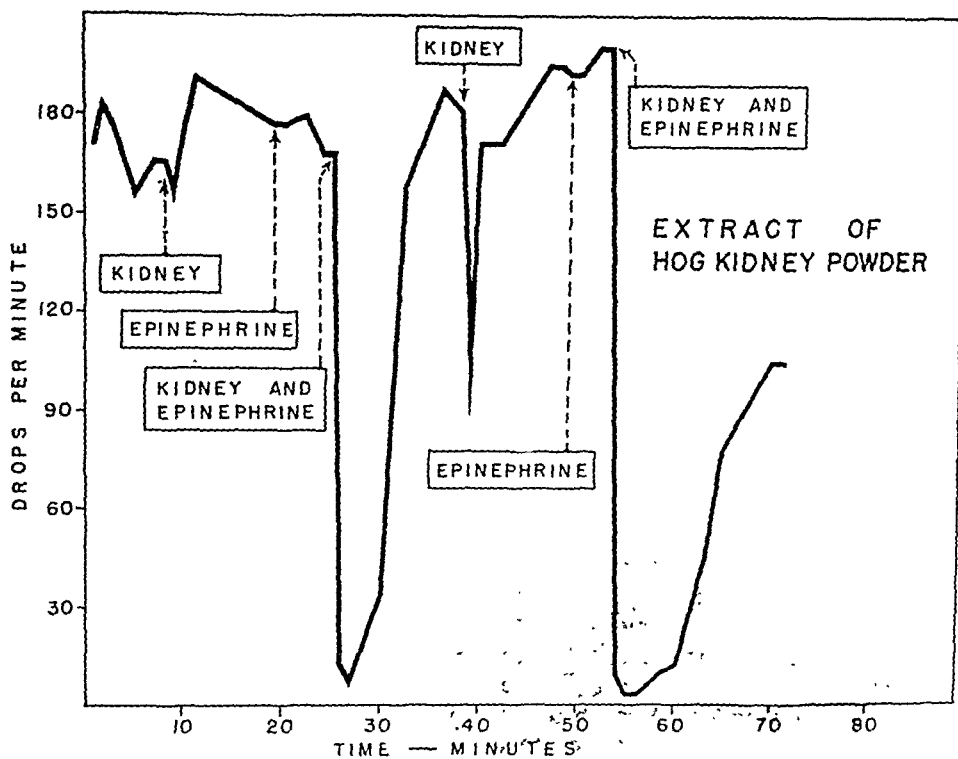
FIG. 1.

Vasoconstrictor action of a combination of an extract of hog kidney powder and epinephrine.
Epinephrine amount used: 1 cc of 1:2,500,000.

was employed since Morton and Tainter[6] did not observe any significant differences between constant and pulsating pressure as tested on the hind leg of the cat. Careful washing out of the ear with Ringer-Locke solution preceded each injection of test material. Epinephrine (Parke, Davis 1:1000) was diluted with distilled water and finally 0.5 cc, 1.0 cc or 1.5 cc of this 1:2,500,000 or 1:5,000,000 solution was made up to 8 cc with Ringer-Locke. The subthreshold dose was determined for each ear and was found to range between .2 and .6 μg. When a mixture of epinephrine and a kidney extract was to be tested, an appropriate amount of Ringer-Locke solution was replaced by kidney extract, the total volume remaining 8 cc.

Hog Kidney Fractions. The simplest of these preparations resulted from the extraction of the dry powder secured by acetone-ether treatment of fresh minced hog kidney. Twenty grams of the powder were extracted with approximately 150 cc of 2% saline; this

suspension was then centrifuged and the supernatant dialyzed.

A second preparation made in this laboratory in 1939[13] resulted from the further treatment of the above saline extract with mono- and dipotassium phosphate and then with 10% sodium chloride at pH 4.0.[14] On intravenous injection 3.6 mg of the resulting protein raised the blood pressure of a 10 kilo dog 30-40 mm of Hg.

A third preparation was made recently by a different method. Aqueous extracts of hog kidney brought to pH 2.9 with 10% trichloracetic acid were filtered, neutralized and fractionated with ammonium-sulfate and alcohol. The procedure is similar to one described for the preparation of renin.[15] 0.36 mg of the

[13] Winternitz, M. C., Mylon, E., Waters, L. L., and Katzenstein, R., *Yale J. Biol. and Med.*, 1940, **12**, 623.

[14] Helmer, P. M., and Page, I. H., *J. Biol. Chem.*, 1939, **127**, 757.

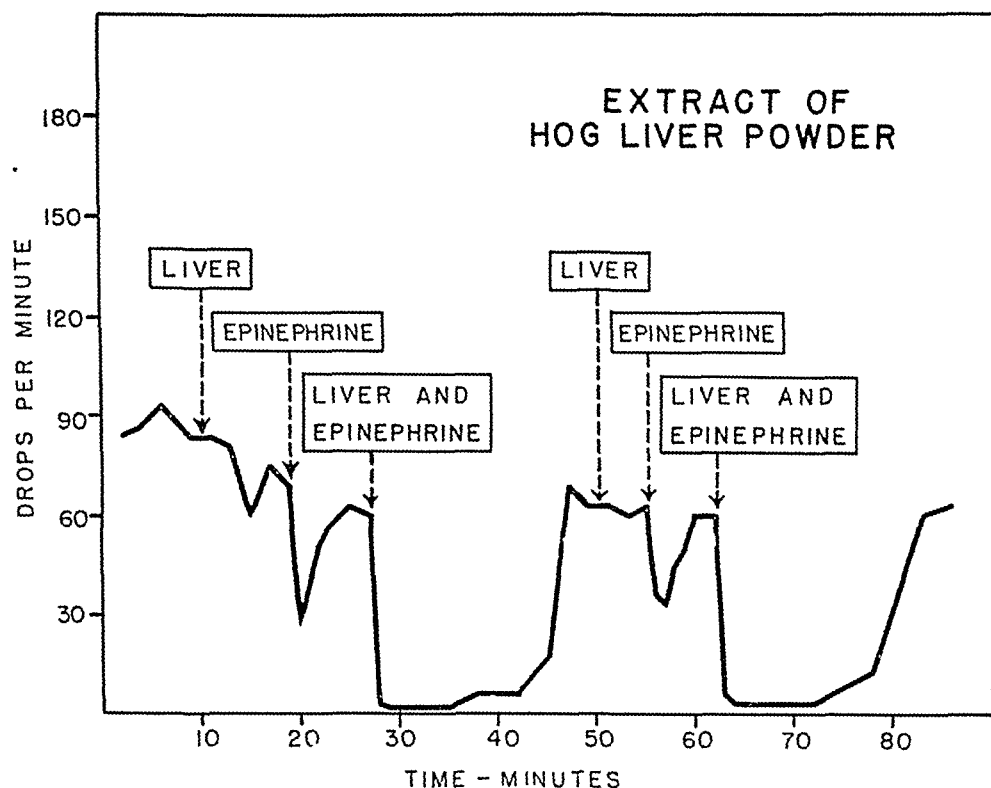[15] Katz, Y. I., and Goldblatt, H., *J. Exp. Med.*, 1943, **78**, 67.

FIG. 1.
Vasoconstrictor action of a combination of an extract of hog liver powder and epinephrine. The epinephrine amount used was 1 cc of 1:2,500,000. It should be added that at least 5 times the amount of epinephrine alone is required to produce an equipressor effect.

of the extract with high activity had a total content of only 0.8 mg of protein.

To corroborate this result in part, 2 more lots of liver were processed after the same fashion detailed above. Despite rigid adherence to the prescribed method, the fractions of the two new lots with highest activity when supplemented with epinephrine were not the same. This is shown in Table I that represents 36 experiments with the various fractions of lots 2 and 3.

Analysis of Fraction IV A. This fraction was lyophilized and no reduction of its vasoactivity resulted. Samples of the lyophilized fraction, a fluffy, light brown powder, were analyzed with the following results:

1. *Qualitative Tests:* The usual qualitative tests for protein were positive. 95% of the nitrogen was amino N. There was a positive test for sulfur.
2. *Quantitative Analysis:* All elements reported were calculated on a moisture and ash free basis.

%H	C	N	S	P*
6.98	49.18	15.18	0.95	0.06

* The phosphorus probably represents an impurity in the ash.

Discussion. Protein fractions of several organs of various species become vasoconstrictive when supplemented with subthreshold quantities of epinephrine just as such addition is known to effect otherwise inactive sympatomimetic amines.[6] Whether this activation

TABLE I.

Activity	Fractions	Protein range, mg cc	Lot No.
None	3	—	2
Moderate	10	0.12 - 5.6	2
Marked	5	2.6 - 13.2	2
None	4	0.5 - 1.7	3
Moderate	3	0.8 - 2.4	3
Marked	6	0.4 - 4.7	3

6 Morton, M. C., and Tainter, M. L., *J. Physiol.*, 1940, 98, 264.

16097

Influence of Epinephrine on Vasoconstrictive Action of Organ Extracts.*

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Epinephrine quantitatively insufficient in itself to have any constrictor effect has been observed to activate various otherwise inert fractions of kidney extract as demonstrated by constriction of the vessels of the perfused rabbit's ear.[1] It has been shown that this effect is neither species nor organ specific. This evidence will now be presented together with such facts as have been secured related to the character of the agent in the tissue extracts.

Organ extracts of hog, dog, and rabbit including liver and heart as well as kidney have been prepared in the following several ways:

(1) Approximately 200 g of minced organ were thoroughly mixed with equal amounts of saline, kept at 5°C for 24 hours prior to removing the precipitate by centrifugation and dialysis of the supernatant fluid.

(2) Twenty g of the dry powder residue after acetone-ether extraction of minced organ, were treated with approximately 150 cc of 2% saline and centrifuged to secure the supernatant for dialysis.

(3) Many methods of fractionating the above more crude extracts were utilized. Only the one that yielded a product of greater interest is detailed. Approximately 5 kg of fresh hog liver were minced. The following procedures were carried out at 5°C: Extraction with 10 liters of distilled water for 24 hours and filtration through several layers of gauze; the careful adjustment of the filtrate to pH 2.9 by slow addition of 10% trichloracetic acid. (The final concentration of the trichloracetic acid was 1.4%.) Removal of the precipitate through fluted filter paper (Whatman No. 2) after 6 hours. The filtrate was then fractionated with ammonium sulphate, the precipitates redissolved and dialyzed, refractionated with ammonium sulphate and alcohol, in general following the procedure described for the preparation of renin from kidney.[2] The obtained organ fractions are of protein nature and differ from the dialysates prepared by Cannon and Lissak,[3] the alcohol extracts used by v. Euler[4] and the preparations made by Raab and Humphreys.[5]

Results. No significant vasoconstriction resulted from the perfusion of the rabbit's ear with either the dialyzed saline extracts of hog heart or liver or with the saline preparation of the powders resulting from acetone-ether treatment of any of the three organs of the several animal species. Even addition of minimal quantities of epinephrine had reasonably little effect as supplement to the saline extract of minced organ. This latter finding contrasted with the result of similar addition of epinephrine to the saline preparation derived from treatment of organ-powder after acetone-ether extraction. Vasoconstriction was always evident and as a rule it was marked. This is illustrated in Fig. 1.

Marked vasoconstrictor effects resulted from the addition of epinephrine in minimal amounts to the last fraction of the liver designated as IV A. A typical experiment is illustrated in Fig. 2.

Throughout this study attention was directed to the ratio of the nitrogen content of the perfusate to the activity curve. The trend had been downward and with Fraction IV A reached the new low. Two cubic centimeters

1 Mylon, E., Horton, F. H., and Levy, R. P., PROC. SOC. EXP. BIOL. AND MED., 1947, 66, 375.

2 Katz, Y. J., and Goldblatt, H., *J. Exp. Med.,* 1943, 67, 78.

3 Cannon, W. B., and Lissak, K., *Am. J. Physiol.,* 1939, 125, 765.

4 v. Euler, U. S., *Nature,* 157, 369, March 23, 1946.

5 Raab, W., and Humphreys, R. J., *Am. J. Physiol.,* 1944, 148, 460.

16098

A Machine for Shell-Freezing Biological Materials in Small Glass Ampoules.

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The increasing use of desiccation *in vacuo* from the frozen state as a method for preserving biological materials has created the need for equipment which will shell-freeze small amounts of fluid in a few ampoules without undue effort and expense. The machine described below is suitable for this purpose. It eliminates the irregularities in thickness of shell which result from rotation of ampoules by hand and the prohibitive cost of a single small run in one of the shelling machines designed for large scale production.[1]

A cylindrical block, or rotor, of duraluminum, 4 inches deep and 5 inches in diameter, constitutes the main portion of the freezing unit (Fig. 1 and 2). Eight drilled holes which have a diameter of 11/16 inches for a depth of $2\frac{1}{2}$ inches provide the spaces into which the ampoules fit with a minimum of play. The holes penetrate the entire block to facilitate cooling, but are reduced in diameter to $\frac{1}{4}$ inches throughout the terminal $2\frac{1}{2}$ inches of their depth, the place at which the diameters change, forms a shoulder upon which the ampoule rests. A small electric motor turns the rotor at 35 r.p.m. through a reducing gear, the shaft of which is directly attached to the duraluminum block by means of a hub and set-screw. The freezing mixture of alcohol and crushed "dry ice" is placed beneath the rotor in a pan in such a manner that only a small segment of the circumference of the rotor is submerged to a depth of no more than $\frac{1}{2}$ inches at its deepest point. The rotor and motor are mounted on a wedge-shaped base so that the outer surface of the rotor has an angle of about 5 with the surface of the chilled liquid.

1 Strumia, M. M., and McGraw, J. J., J. Lab. and Clin. Med., 1943, 28, 1140.
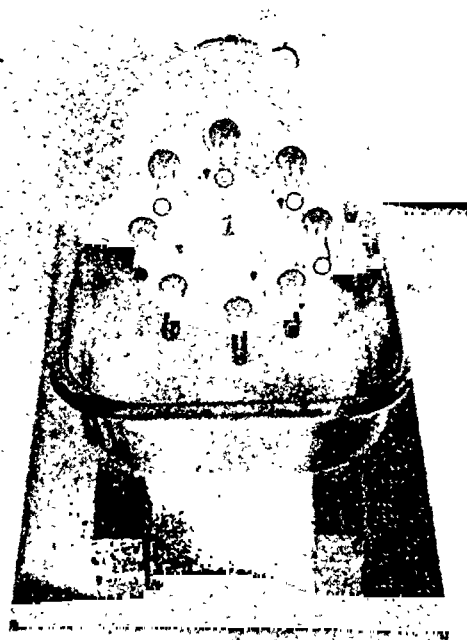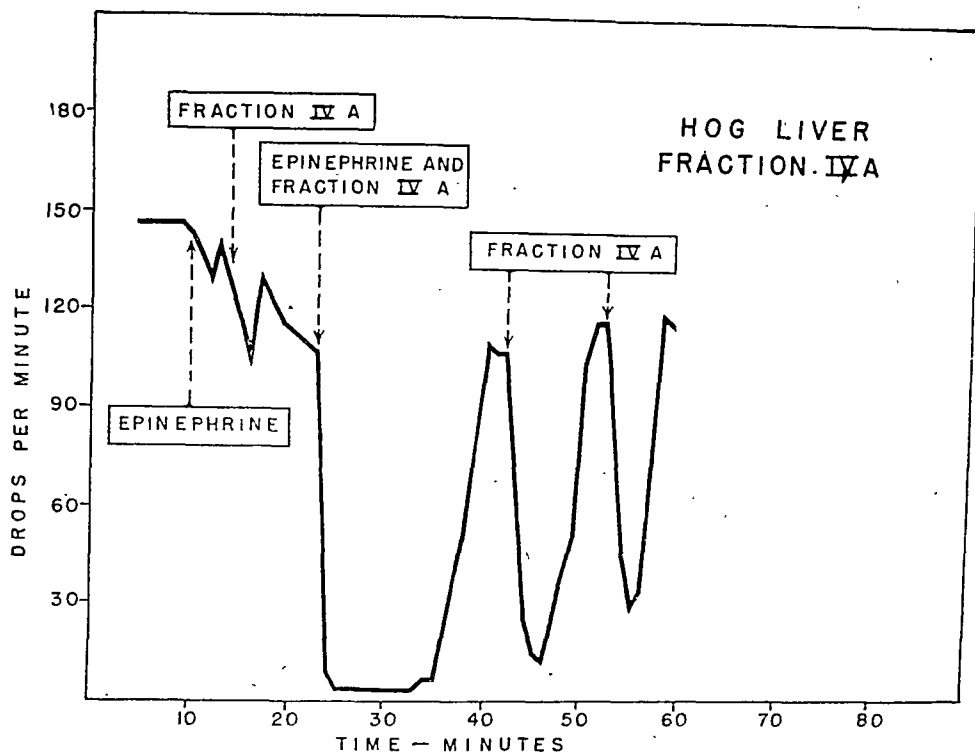

Fig. 1.
A machine for shell-freezing biological materials in small glass ampoules.

In operation, the rotor is first chilled by rotating for five minutes in the freezing bath and the cotton stoppered ampoules are then rapidly inserted in the holes. Approximately one minute is required to freeze an ampoule containing 3 cc of fluid. Approximately 400 ampoules can be processed in an hour with the apparatus illustrated. The unit is particularly useful for freezing small lots, such as one-half to 3 dozen ampoules; for this about 3 to 4 pounds of "dry ice" are required.

Summary. An apparatus is described for the rapid shell-freezing of fluids in small containers preparatory to drying from the frozen state (lyophilization).

indicates a potentiation of epinephrine or a stimulation of effector cells normally responding to this compound remains unanswered.

Varying amounts of phospholipid were removed from the protein fraction by the different methods employed for separation. This may be associated with the change of the vasoconstrictor effect on blood vessels.

Summary. Many protein fractions isolated from kidney, liver and heart of hog, and from kidney and liver of dog and rabbit, inactive when perfused alone through the rabbit's ear, become vasoconstrictive at varying degrees when combined with subthreshold quantities of epinephrine.

In contrast, dialyzed saline extracts of fresh minced organs were inactive with and without addition of epinephrine. After freezing and thawing or prolonged dialyzation against distilled water epinephrine addition resulted in some activity. 0.8 mg of protein or protein mixture secured by continued fractionation of hog liver and combined with subthreshold amounts of epinephrine, produced marked and long lasting vasoconstrictions.

and the recognition of certain deficiency syndromes in the chronic alcoholic patient,[6,7] there has been a progressive tendency to attribute all of the organic changes in alcoholism to the supposed universal dietary deficiency.[8]

The observations of Connor,[9] however, are in opposition to this and should be noted. In rabbits, he was able to produce fatty infiltration, liver cell atrophy, necrosis of liver cells, and fibrosis in variable degree by daily administration of alcohol, even though a diet with high casein content and an adequate amount of other known required factors were provided. The use of the rabbit, an animal in which perilobular fibrosis and parasitic disease of the liver are common findings, the fact that the animals did not take an adequate amount of the diet, and the lack of control animals, make it difficult to assess fully the findings in Connor's experiment.

The problem has therefore been undertaken in rats. Adult female rats of the Sprague-Dawly strain were used. Four series were employed:

Group   I—Low casein diet,* and alcohol,
Group   II—High casein diet, and alcohol,
Group III—Low casein diet, no alcohol, and
Group   IV—High casein diet, no alcohol.

The administration of alcohol caused a reduction of food intake. The amount of food

5 Du Vigneaud, V., *Biol. Symposia*, 1941, 5, 234.
6 Alexander, Leo, *Am. J. Path.*, 1940, 16, 61.
7 Goodheart, R., and Jolliffe, N., *Am. Heart J.*, 1938, 15, 569.
8 Wright, A. W., *Arch. Path.*, 1941, 32, 670.
9 Connor, C. L., *Arch. Path.*, 1940, 30, 165.

* The composition of the diets[10] employed was:

	Low casein	High casein
Lard	40%	40%
Agar	2	2
Starch	48	28
Salt mixture	48	28
Osborne, Mendel	5	5
Casein	5	25

Each animal was given daily one drop of Vi Penta furnished through the courtesy of Hoffmann-LaRoche), each 0.6 cc of which contains 5000 USP units of vitamin A, 1 mg of thiamine HCl, 1 mg of riboflavine, 50 mg of ascorbic acid, 1000 USP units of activated ergosterol, and 2 mg of niacinamide.

10 Tidwell, H. C., and Treadwell, C. R., *J. Biol. Chem.*, 1946, 162, 155.

administered was therefore placed under control by allowing Groups I and II to eat *ad libitum,* and the amount taken was determined by weighing the food containers. The amount of food given to Group III and IV was the same as that eaten by the corresponding group taking alcohol, and on the preceding day. Alcohol was administered daily by stomach tube, the amount of 1.5 cc of 25% alcohol per 100 g, and 10% alcohol was given as the only drinking water. Approximately 0.6 cc of absolute alcohol per 100 g of body weight was taken daily by these animals. The regime was continued for about 2 months, and at the end of this time the animals were autopsied and histological study of the livers and other organs made.

Results. The rats receiving alcohol became obviously intoxicated and not infrequently were comatose for short periods following the administration of alcohol. These animals ate poorly. The groups receiving no alcohol continued to exhibit good appetite throughout, although the animals receiving low casein diet later became slightly less inclined to eat their food.

The weight declined in all groups. Fig. 1 and 2 show the decrease for each animal.

Fatty livers were found in every animal of Groups I, II and III. No fatty change was found in Group IV. The gross and histological findings were characteristic of this degenerative lesion. The liver was increased moderately in weight and was somewhat yellowish and friable. Histological study of hematoxylin-eosin and Sudan stained sections revealed fatty deposition in the liver cells and was moderate or marked, predominantly the latter. No evidence of cirrhosis was found.

Discussion. In these experiments, the effect of emaciation from quantitative nutritional lack has been eliminated by providing the same amount of food to the corresponding control and alcohol groups. The weight curves further establish this point, since the group receiving alcohol and adequate diet, showed slightly less weight decline than those receiving no alcohol and quantitatively adequate diet. Thus any liver cell injury might well
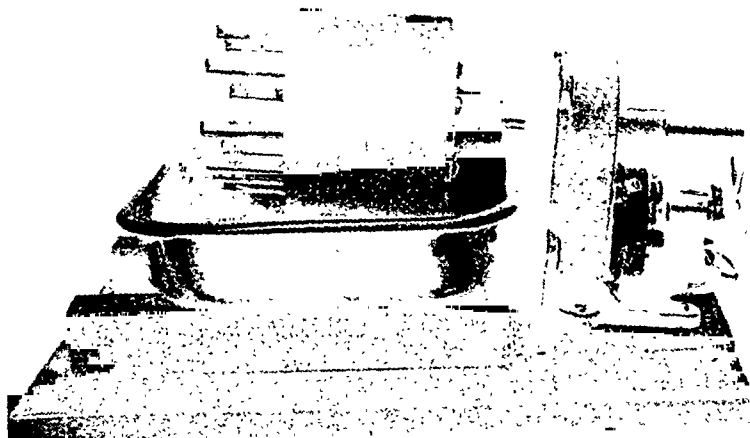
FIG. 2.
A machine for shell-freezing biological materials in small glass ampoules.

16099

Production of Fatty Infiltration of Liver in Rats by Alcohol in Spite of Adequate Diet.

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The part played by alcohol in liver cell injury and cirrhosis has not been conclusively established. Prior to the recognition of the influence of dietary factors in liver injury, many observations were made in an attempt to ascertain the solution to this question. Moon[1] summarized and critically evaluated these reports, concluding that there was no definite evidence that alcohol itself produced liver cell injury. At the time of Moon s review, 1934, the dietary factor was not given any consideration, and its significance was not

apparently recognized. Lamson and Wing[2] showed that the combination of alcohol with carbon tetrachloride increased the severity and incidence of liver cell injury and cirrhosis. This observation was evidently acceptable to both sides of the question as a compromise and seemed to constitute the basis for prevailing opinion on the subject until recent years. Following the elucidation of the dietary factor in the problem of liver cell injury,[3,4,5]

1 Moon, V. H., *Arch. Path.*, 1934, **18**, 381.

2 Lamson, P. D., and Wing, R., *J. Pharm. and Exp. Therap.*, 1926, **29**, 191.

3 Allen, F. N., Bowie, D. J., McLeod, J. J. R., and Robinson, W. L., *Brit. J. Exp. Path.*, 1924, **5**, 75.

4 Best, C. H., and Huntsman, M. E., *J. Physiol.*, 1935, **83**, 255.
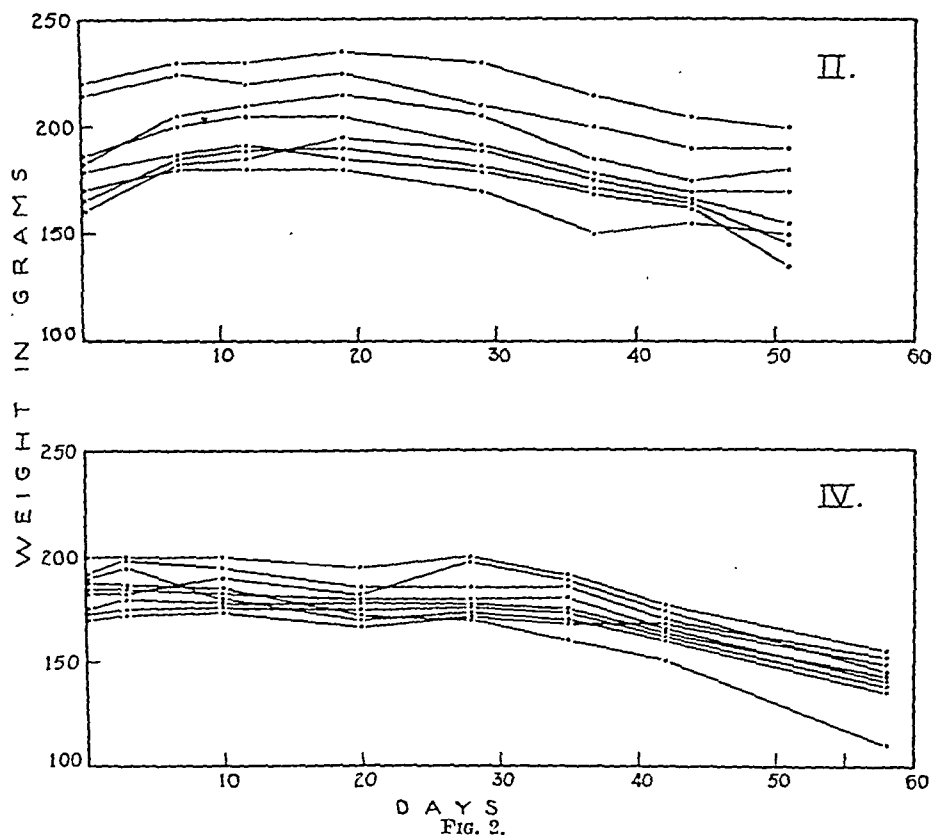
WEIGHT   CURVES   ON   GROUPS II AND IV

FIG. 2.

which permits accumulation of fat within the liver cells, and that this effect operates separately from that of extrinsic deficiency of lipotropic factors.

16100

Complement-Fixation in Experimental and Human Poliomyelitis.[*]

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Although many attempts have been made to find a simple immunological means of studying poliomyelitis. only the neutralization reaction has yielded consistent and reliable results. Possible reasons for the lack of success of precipitin and complement-fixation tests have been discussed by several authors.[1] The possibilities include (a) low reactivity or avidity of the neutralizing antibodies, (b) small size of the virus particle, and (c) insufficient concentration of virus in the antigen

1 Schultz, E. W., Gebhardt, L. P., and Bullock, L. T., *J. Immunol.*, 1931, 21, 171; Raffel S., and Schultz. E. W., *J. Immunol.*, 1940, 39, 256.
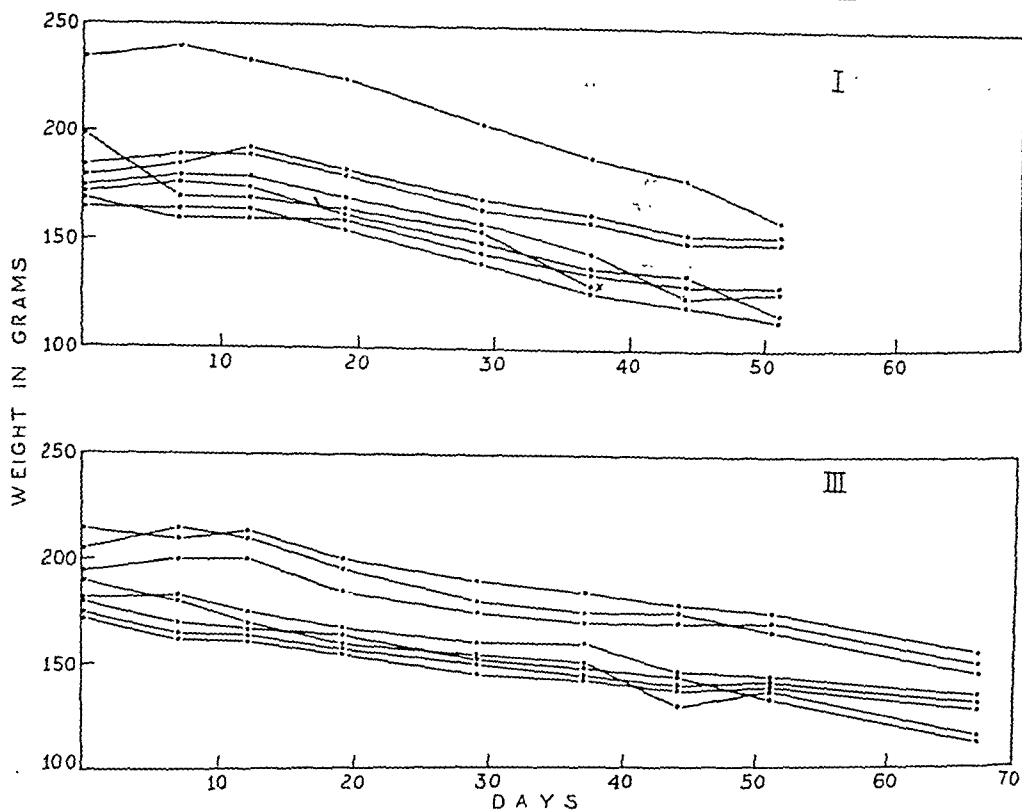
WEIGHT CURVES ON GROUPS I AND III

FIG. 1.

be attributed to the only apparent variable, namely the presence or absence of alcohol. The fact that in Group II, receiving high casein diet and alcohol, fatty infiltration of the liver occurred in all animals, would seem therefore to indicate that alcohol itself does produce liver cell injury, and in this instance, fatty infiltration. There was no apparent difference in the incidence or severity of fatty degeneration nor is there any histological difference in the character of the lesion in the animals receiving alcohol and high casein diet, as compared to those receiving low casein diet with or without alcohol. The duration of the period of observation was too short to determine the occurrence of cirrhosis in these animals. It remains to be shown whether the liver cell injury produced by alcohol is due to a direct toxic action, to the effect of the degradation products of alcohol,

or whether alcohol interferes with the chemical processes normally called into play in the metabolism of fat.

Summary. Rats were given alcohol daily and either a high or low casein diet Control series received similar diets and no alcohol. The amount of food intake in control groups was regulated according to the amount taken by the rats receiving alcohol. The effect of total food intake in those receiving alcohol as compared with those not receiving alcohol was further observed by weight curves. Fatty infiltration of the liver occurred in every animal receiving alcohol and low casein diet, alcohol and high casein diet. and in all those receiving low casein diet and no alcohol. None of the animals receiving the same volume of the high casein diet and no alcohol had fatty infiltration of the liver. It is therefore concluded that alcohol exerts an effect

cubated at 37°C for one hour, set in the refrigerator for 2 hours or slightly more to permit settling of cells, and read. The last tube showing 50% lysis was taken to determine the titer. Titer is expressed in these experiments as the number of units of complement-fixing antibody contained in one ml of undiluted serum. It is worthy of emphasis that in carrying out these tests the following precautions were observed: (a) The tubes were incubated at low temperature for eighteen hours to assure maximum reaction and minimal deterioration of complement. (b) The antigen was tested for possible anticomplementary properties by incubation with fractional units of complement in advance of and simultaneously with each group of tests. (c) A control for anticomplementary properties of each serum was made throughout the dilution range simultaneously with the actual test.

The data presented in Table I show that complement-fixing antibodies against active virus were present to a variable degree after immunization with formaldehyde-inactivated virus. As both the method of vaccination and the elapsed time before blood was drawn varied in these experiments, it was reasonable to expect the type of variation found. When blood was drawn about three weeks after the last injection of vaccine in the animals which were refractory to infection, Group c, uniformly high titers of >80 were found. Blood from vaccinated animals which developed poliomyelitis but recovered with some residual signs of the disease. Group a, also showed similar high titers. Samples taken five months after immunization, however, (Group b) gave relatively low titers although these animals had been given several injections of crude 20% infected-brain extract a few weeks previously.

As the vaccine used for immunization in the above mentioned experiments had been obtained from a virus preparation that may have contained some normal rat tissue proteins, the positive results could be explained by immunization to the normal tissue components as well as by immunization to virus. In order to study this question, the complement-fixing

TABLE II. Complement-fixation of Immune Sera with Concentrated Virus and Normal Tissue Antigen.

Degree of complement-fixation at serum dilutions and with antigens indicated

Immunization history	Sera	Virus; serum 1:								Normal; serum 1:								Normal 1/10; serum 1:								Serum control					Titer for Virus	Titer for Normal	Titer for normal 1/10
		0	2	4	8	16	32	64	128	0	2	4	8	16	32	64	128	0	2	4	8	16	32	64	128	0	2	4	8			
Group a. Pooled sera from 6 vaccinated rats which proved resistant to subsequent challenge*	Fresh	4	4	4	4	4	4	4		2	3	3	2	0	0	0	0	1	1	1	0	0	0	0	0	±	±	±	±	>320	80	+
	Frozen 7 days	4	4	4	4	4	4	3	±	3	4	4	4	2	0	0	0	3	3	2	1	0	0	0	0	±	1	±	0	640	160	40
Group b. Four vaccinated rats which proved resistant to subsequent challenge†	Fresh	4	4	4	4	4	0	0	0	4	4	4	2	0	±	0	0									+	0	0	0	160	80	
		4	4	4	4	4	0	0	0	4	4	3	3	±	0	0	0									±	0	0	0	160	80	
		4	4	4	4	4	0	0	0	4	4	4	4	2	0	0	0									0	0	0	0	>80	80	
		4	4	4	4	2	0	0	0	4	4	4	4	0	0	0	0									0	0	0	0	>320	160	

* Injected with 10^{-5} g formalized virus nitrogen. Preparation 91-2-4:3 as follows: 2.5×10^{-6} g intradermally on 1/24/47; 5×10^{-6} intramuscularly on 1/31/47 and 2.5×10^{-6} g intradermally on 2/7/47. Challenged on 2/17/47. Bled 3/10/47.

† Same group of rats as Group c, Table I. Bled 4/8/47.

Virus preparation 91-2-4:3 used at 10^{-5} g nitrogen per ml; preparation of normal tissue components used at 10^{-5} g nitrogen per ml and at 10^{-6} g nitrogen per ml as indicated.

TABLE I.
Complement-fixation with Concentrated Lansing Virus and Sera from Immune Rats.[*]

Immunization history	Rat No.	Serum 1:				Serum control	Titer (units per ml)
		0	2	4	8		
Group a, vaccinated rats which developed "polio" on challenge, but survived with residual paralysis.	66	4	4	4	3	0	80
	67	4	2	±	±	0	20
	94	4	4	4	2	0	80
Group b, vaccinated rats which proved resistant to subsequent challenge .	90	2	2	±	±	0	20
	91	1	1		0	0	+
	92	±	0	0	0	0	0
Group c, same as group b, vaccinated with a different preparation of formalized virus	63		4	4	4	0	>80
	64	4	4	4	±	0	>40
	95		4	2	4	0	>80
	100			4	4	0	>80
	102	4	4	4	4	0	>80
	107		4	4	4	0	>80
	113	4	4	4	4	0	>80
	116	4	4	4	4	0	>80

Groups a and c. Injected with 10-5 g formalized virus nitrogen, Preparation 91-2-4,[3] as follows: 2.5 x 10-6 g intradermally on 12/16/46, 5 x 10-6 g intramuscularly on 12/23/46, and 2.5 x 10-6 g intradermally on 12/30/46. Challenged on 1/6/47. Bled 1/21/47.

Group b. Injected with 3 x 10-6 g formalized virus nitrogen, Preparation 87[3] as follows: 2 x 10-3 g on 8/15/46 and 10-6 g on 8/20/46 intraperitoneally. Challenged 8/24/46. Rechallenged 9/27/46. Reinjected with 0.5 cc of 20% crude virus suspension twice weekly from 10/12/46 to 1/2/47. Bled 1/21/47.

[*] Virus preparation 91-2-4[3] at 2 x 10-5 g nitrogen per ml used as antigen; antigen control with one unit complement = 0, with ½ unit complement = 2, and with no complement = 4.

preparations used.

Recent work on the concentration of the Lansing strain of poliomyelitis virus[2] has provided more concentrated virus preparations than those previously employed in immunological tests. In a previous paper[3] the immunization of cotton rats with formalized preparations of highly concentrated Lansing virus and their protection against the intracerebral injection of large doses of active virus was reported. If the high degree of immunity produced in many of these animals was due to a high titer of humoral antibodies, it seemed possible by the use of concentrated virus as antigen that positive precipitin or complement-fixation tests could be demonstrated in such sera. Precipitin tests were attempted but proved negative. Complement-fixation tests, on the contrary, proved definitely positive.

The results obtained with sera from 2 groups of rats that had been injected with formalized virus and had proved refractory to relatively large amounts of active virus,

as well as with sera from 3 immunized rats which developed poliomyelitis on subsequent challenge, are given in Table I. The complement-fixation tests were made as follows: The immune sera were incubated at 56°C for 20 minutes to destroy the complement present and mixed with saline to give the dilutions used. A 0.1 ml volume of each dilution was then distributed in small test tubes, to which was added 0.2 ml of a dilution of guinea pig complement containing 2 units, 0.6 ml of saline, and 0.1 ml of a 2-cycle virus preparation containing 10-5 g nitrogen per ml. Preliminary tests showed this concentration of virus to be devoid of anticomplementary properties when tested with fractional units of complement. Controls without antigen were set up for the entire range of dilutions of the sera. Controls for possible anticomplementary action of the antigen were also set up along with each set of tests. The tubes were shaken and incubated at 4°C overnight. To a 4% suspension of sheep red blood cells was added an equal volume of solution containing eight units of hemolysin per ml and 0.5 ml of this suspension of sensitized cells was added to each test tube. The tubes were shaken, in-

2 Loring, H. S., and Schwerdt, C. E., PROC. SOC. EXP. BIOL. AND MED., 1946, 62, 291.

3 Loring, H. S., Schwerdt, C. E., Lawrence, N., and Anderson, J. C., Science, 1947, 106, 104.

TABLE III.

Complement-fixation of Normal Rat Serum with Virus and with "Normal" Antigen.

History of animals	Virus preparation	Virus; Serum 1:						"Normal",* Serum 1:						Serum control						Titer for virus	Titer for "Normal"
		0	2	4	8	16	32	0	2	4	8	16	32	0	2	4	8	16	32		
Group a. 8 wks old, kept in animal room 3 weeks before blood samples taken	91.2-4	3	3	3	1			0	0					0	0	0	0			40	0
	96-7-8	3	3	3	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	40	0
	,,	2	2	1	±	0	0	2	1	1	±			1	1	1	1	0	0	20	±
	,,	2	4	4	±	0	0	1	2	±	±			0	0	0	0	0	0	40	20
Group b. 5 wks old, bled when received from dealer	,,	2	3	2	0	0	0	2	3	3	±	0	0	0	0	0	0	0	0	40	20
	,,	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+	0
	,,	3	2	4	4	±	0	3	4	4	4	3	0	0	0	2	3	0	0	80	80
	,,	4	4	4	3	0	0	4	4	4	±	±	0	0	0	1	0	0	0	80	>40
	,,	±	4	3	0	0	0	3	1	2	0	0	0	0	0	0	0	0	0	40	>40
	,,	4	4	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	+
	,,	4	4	3	3	±	±	0	3	4	4	2	2	0	0	0	0	0	0	80	0
	,,	4	4	4	±	±	0	3	4	4	3	±	0	0	0	0	0	0	0	80	90
	,,	2	4	4	±	±	0	0	1	2	±	±	0	0	0	0	0	0	0	80	>40
	,,																				40
Group c. Pooled sera from 3 rats 5 wks old	100	0	0	0	0	0	0	0	0					0	0	0	0	0	0	0	

* "Normal" antigen = preparation from normal brain and spinal cord. Virus preparations 96-7-8 and 100 were prepared as previously described and were used at a concentration of 10^{-5} g nitrogen per ml.

titer of each serum was determined with a similarly concentrated preparation of normal cotton rat brain and spinal cord in comparison with that of concentrated virus.

A total of 257 g of brains and spinal cords from normal cotton rats were processed by the procedure used for the preparation of the virus concentrate.[2] After 2 ultracentrifugal cycles of purification, the high molecular weight sediments obtained contained 0.85 mg of nitrogen. The yield of nitrogen found in this experiment was relatively higher than those obtained from normal tissue after 3 or 4 cycles of purification as previously reported. This normal constituent was used in the complement-fixation tests at the same concentration as that used for the virus preparation, namely 10^{-5} g nitrogen per ml, and also at one-tenth this concentration.

The results of this experiment are summarized in Table II. It may be seen that a sample of pooled immune serum from 6 immunized and refractory rats fixed complement when either the concentrated virus or the preparation from normal rat tissue was used as antigen. In all the tests made, however, the titer for virus (160 to 1280) was significantly higher than that for the normal preparation at either the same concentration, (titers of 80 to 160) or at one-tenth the concentration (titers of + to 40). Because of the difference in titers with the 2 antigens, the results suggest that complement-fixation in the presence of virus was due primarily to the reaction of antibody with virus rather than to a reaction of antibody with a normal component present in the virus preparation. Another possible explanation for positive complement-fixation with the antigen from normal tissue, in addition to that mentioned above, is that poliomyelitis virus produced in cotton rats may be related antigenically to certain normal brain tissue proteins. The latter phenomenon would be similar to that observed with several infectious agents and certain of the normal antigens of the host.[4,5,6]

Experiments were conducted with sera from normal rats to determine if antibodies were present which would fix complement in the presence of either virus or the normal tissue preparation. These results are summarized in Table III. It may be seen that complement-fixation occurred with most of the sera tested with virus as antigen in animals either 5 or 8 weeks old. Less consistent results and in general lower titers were found with the "normal" antigen. Several instances are recorded where positive tests were found with virus, but no antibodies to the "normal" antigen could be detected. These results are similar to those found by Casals and Palacios[7] and by Kidd and Friedewald[8] with non-specific antibodies occurring in normal serum. In the latter instances it was reported that the non-specific antibody could be eliminated by heating the serum to a higher temperature than that usually employed to destroy complement. It was of interest to determine if the reaction with the virus preparation and the "normal" antibody could be similarly eliminated. Two normal sera which fixed complement in the presence of virus after they had been heated at 56°C for 20 min. were heated for an additional 20 min. at 60°C and again tested with the virus concentrate as antigen. In both cases negative tests were now found indicating that the normal antibody had been eliminated by this method.

The same procedure was next applied to the 5 immune sera shown in Table II which had fixed complement in the presence of either virus or the "normal" antigen. After they had been heated 20 min. at 60°C, they were again tested with virus and with the "normal" antigen. In 4 of the 6 samples the reaction with the "normal" antigen was eliminated whereas positive complement fixation (titer of 20 to 80) was still found with the virus concentrate.

A consideration of the results obtained in all the experiments mentioned seemed to justify the conclusion that complement fixa-

4 Duran-Reynals, F., *Yale J. Biol. and Med.*, 1940, **13**, 61.

5 Chambers, L. A., and Henle, W., *Am. J. Path.*, 1941, **17**, 442.

6 Knight, C. A., *J. Exp. Med.*, 1946, **83**, 281.

7 Casals, J., and Palacios, R., *J. Exp. Med.*, 1941, **74**, 409.

8 Kidd, J. G., and Friedewald, W. F., *J. Exp. Med.*, 1942, **70**, 543.

TABLE V.
Comparison of Neutralization and Complement-fixation Tests with Human Sera.

Serum	Dilution used	I.D. 50*	Neutralization titer†	Complement-fixation titer
M.K.	Convalescent	1:1000	10-7.9	10-4.9	320
R.He.	,,	1:100	10-9.0	10-7.0	+
D.W.	,,	,,	10-8.8	10-6.8	0
M.P.	Fatal case	,,	10-10.3	‖10-8.3	0

* 1 I.D. 50 = g of virus nitrogen required in a dose of 0.05 ml to infect 50% of animals when the dilution of serum given was used.
† Neutralization titer is defined as grams of virus nitrogen neutralized to the 50% end point by 1 ml of undiluted serum.

itive tests with titers that were comparable to those obtained the first time.

A comparison of the histories of the patients failed to show any correlation of positive or negative reactions with either age of the patient or length of time between onset of the disease and the testing of blood samples. The demonstration that approximately 45% of the group of patients possessed humoral antibodies that react with Lansing virus emphasizes the importance of this or a closely related strain as one responsible for poliomyelitis in humans. In the case of the negative reactions it is possible either that the individuals in question failed to produce humoral antibodies in sufficient concentration to be detected or that they may have been infected by a poliomyelitis virus immunologically unrelated to the Lansing strain.[9]

As shown in Table IV the samples of convalescent human sera showed a wide range in their complement-fixing titers. It was of interest to determine if complement-fixation titer could be correlated with ability to neutralize active virus. Neutralization tests were accordingly made on four human sera, namely the one giving the strongest reaction (M.K.), one showing weak complement-fixation (R. He.), and 2 giving no reaction (D. W. and M. P.). The tests were set up using a 1:100 or a 1:1000 dilution of serum in saline with serial dilutions of virus in steps of ten from 10-11 g nitrogen per ml to 10-5 g nitrogen per ml. The mixtures were incubated at 37° for one-half hour, and 0.05 ml of each dilution was injected intracerebrally

into each of 5 young cotton rats. The infectivity of the mixtures was determined by the incidence of poliomyelitis and the amount of active virus necessary to infect 50% of the animals calculated according to the method of Reed and Muench.[10] The results which are recorded in Table V showed that over a hundred times as much virus was neutralized by serum M. K. (complement-fixation titer 320) as by either the weakly positive or the negative convalescent sera. Of interest was the fact that the sample from the fatal case, M. P., neutralized less than one-tenth as much virus as the negative convalescent serum. These results are, therefore, in agreement with the view that the same antibody was involved in complement-fixation and in virus neutralization.

Conclusions. The experiments presented above show that complement-fixing antibodies can be demonstrated in the blood of rats immunized with formalized Lansing virus, in convalescent monkey serum, and in some convalescent human sera when concentrated Lansing virus from cotton rats is used as antigen. The results with rat sera are complicated by the presence of antibodies against components of normal brain and spinal cord in both immune and normal animals, but the evidence indicates that the primary reaction with immune sera is between antibody and virus. Immunization as a result of injection of formalized virus is, therefore, associated with the production of serologically detectable antibodies to active virus. The experiments with the convalescent monkey and human

9 Sabin, A. B., *J. Am. Med. Assn.*, 1947, **134**, 749.

10 Reed, L. J., and Muench, H., *Am. J. Hygiene*, 1938, **27**, 493.

TABLE IV.

Complement-fixation Tests on Human Convalescent Sera with Concentrated Lansing Virus and Normal Cotton Rat Tissue Antigen.

Name	Case history*	Date of blood sample	Antigen†	0	2	4	8	16	32	64	Serum Control	Titer
M.K.	♀, 23 yr, A.P. 8-1-46	1-28	Virus				4	4	1	0	0	>160
		,,	,,		4	4	4	4	2		0	320
		,,	Normal	0	0	0	0	0			0	0
		3- 5	Virus	4	4	4	4	4	3		0	320
		,,	Normal	0	0	±	0	0	0		0	0
J.I.	♀, 10 mo., A.P. 7-20-46	1-28	Virus	2	4	4	4	2	0		0	160
		,,	Normal	0	0	0	0	0	0		0	0
		3-5	Virus	1	3	3	1	0	0		0	40
		,,	Normal	0	0	0	0	0	0		0	0
J.M.	♂, 28 yr, A.P. 10-11-46	1-28	Virus	2	4	4	2	±	0		0	80
		,,	Normal	0	0	0	0	0	0		0	0
		3- 5	Virus	1	2	2	1	0	0		0	40
		,,	Normal	0	0	0	0	0	0		0	0
R.He.	♂, 33 yr, A.P. 9-7-46	1-28	Virus	1	1	1	1	1	±		0	±
R.H.	♂, A.P. 8-46	,,	,,	1	1	1	1	±	±		0	+
S.McI.	♀, 4 yr, A.P. 8-13-46	,,	,,	±	±	±	±	±	±		0	0
N.K.	♀, 28 yr, A.P. 8-16-46	,,	,,	0	0	0	0	0	0		0	0
F.O'C.	20 yr, A.P. 9-16-46	,,	,,	0	0	0	0	0	0		0	0
N.W.	♂, 21 yr, A.P. 6-5-46	,,	,,	0	0	0	0	0	0		0	0
D.W.	♀, 4 yr, bulbo-spinal poliomyelitis, 10-1-46	,,	,,	0	0	0	0	0	0		0	0
F.C.	♂, 10 yr, A.P. 7-15-46	,,	,,	0	0	0	0	0	0		0	0
G.J.	♂, 26 yr, A.P. 7-15-46	,,	,,	±	±	±	±	±	±		0	0
G.H.	♂, 6 yr, A.P. 9-1-46	,,	,, , 100	1	2	3	2	0			0	80
M.P.	♂, 7 yr acute polio-myelitis 2-4-47, expired 2-5-47	2-4	,, 96-7-8	0	0	0	0	0	0		0	0
		,,	Normal	0	0	0	0	0	0		0	0

* A.P. = Anterior poliomyelitis.

† The virus preparation used, with the exceptions stated, was 91-2-4; the normal cotton rat antigen was the same as that described in the text.

tion had been demonstrated between virus and its antibody. However, unequivocal proof was provided when sera from a Lansing convalescent rhesus monkey and from 13 convalescent human cases† were examined for complement-fixing antibodies with concentrated Lansing virus and with the normal brain and spinal cord concentrate from cotton rats.

Blood was obtained from the monkey 3 weeks after it developed a partial paralysis following intracerebral inoculation with Lansing virus. The serum fixed complement when concentrated virus was used as antigen, giving a titer of 320. No fixation occurred when the concentrate from normal rat tissue was used as antigen, and sera from 6 normal

† The samples of monkey blood were kindly provided through the cooperation of Professor E. W. Schultz and Dr. S. C. White and the human convalescent sera by Drs. H. K. Faber, L. A. Luz, and R. D. Cutter.

monkeys were negative against both the virus and the antigen from normal rat tissues.

The results with 13 recent human convalescent sera and with that from a fatal case are recorded in Table IV. In those cases where positive complement-fixation was found, the tests were repeated several times with essentially identical results. Six of the 14 sera were positive, giving titers that ranged up to 320, seven were negative as was also that from the fatal case obtained the same day final diagnosis was made. In the positive tests there was no question about complement-fixation with a normal brain protein impurity in the concentrated virus since negative results were found when the preparation from normal rat tissues was used as antigen.

Second samples of blood from three subjects giving high titers were reexamined about 5 weeks after the first samples had been tested. As shown in Table IV, these again gave pos-

Tail   Head

FIG. 1.

Rat No. 32, right side, before Pyribenzamine. Tail: India ink plus saline. Head:
India ink plus 1% hyaluronidase.

Note: Dark spot upper center is due to the subsequent injection of Pyribenzamine.

is somewhat uncertain. Cullumbine[8] has investigated the influence of antihistaminic substances on the action of leukotaxine; he could not detect any anti-inflammatory activity.

On the other hand, various inflammatory processes produced by strongly invasive micro-organisms have been associated with the release, from these organisms, of a specific enzyme, namely hyaluronidase (Duran-Reynals[9]). So far, very little is known concerning the role which this enzyme plays in non-bacterial inflammations of the skin. Since relatively large concentrations of hyaluronidase occur in the normal skin, this may indicate a more general function. (Meyer et al.,[10] Meyer[11]) Certain of the observations described below indicate that hyaluronidase may be associated with certain phases of skin inflammations, especially of allergic non-bacterial nature.

We have directed our investigations in this direction and have studied the influence of Pyribenzamine and antistine upon the activity of hyaluronidase both as a spreading factor and as a possible factor responsible for allergic inflammation of the skin.

Experimental. A. Spreading of India ink in rats. The effect of Pyribenzamine and antistine upon the diffusion of India ink in the skin of albino rats in the presence and absence of hyaluronidase was investigated according to the method described by Duran-Reynals[8] and Cahen and Granier.[12] One hundred and twenty male and female rats, each
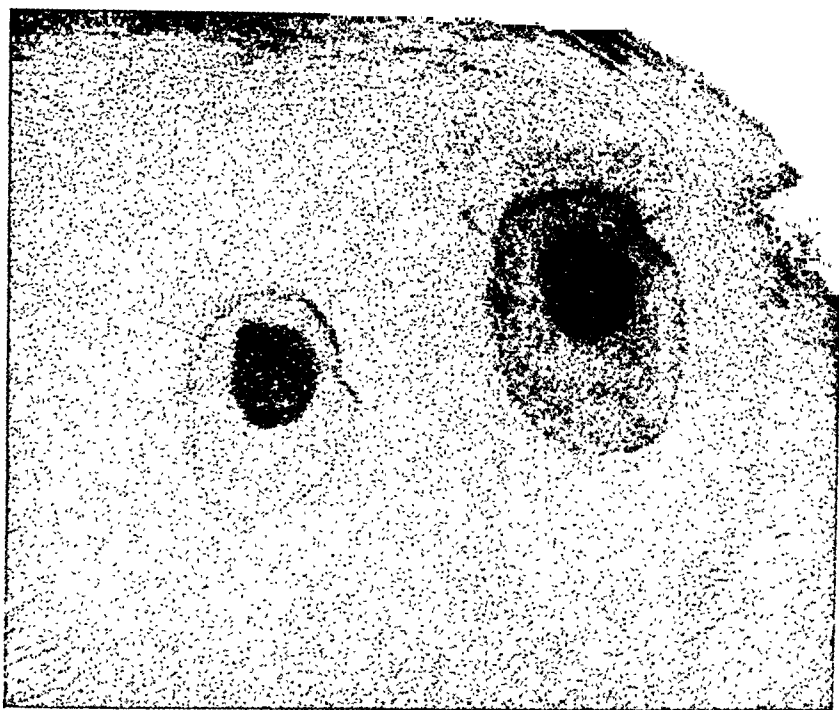
8 Cullumbine, H., *Nature*, 1947, **159**, 841.

9 Duran-Reynals, F., *Bact. Rev.*, 1946, **6**, 197.

10 Meyer, K., Chaffee, E., Hobby, G. L., and Dawson, M. H., *J. Exp. Med.*, 1941, **73**, 309.

11 Meyer, K., *Physiol. Rev.*, 1947, **27**, 335.

12 Cahen, R. L., and Granier, M., *Yale J. Biol. and Med.*, 1944, **16**, 257.

sera provide definite proof that positive complement fixation can be demonstrated when concentrated Lansing virus prepared as described[2] is used as the antigen. These latter results further support the importance of the Lansing virus or an antigenically related strain as one responsible for the human disease.

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Influence of Pyribenzamine and Antistine upon the Action of Hyaluronidase.

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It is the general belief that the antianaphylactic and anti-allergic properties of Pyribenzamine and similar substances are due primarily to their ability to nullify the pathologic effects of histamine. This belief is based upon the following premises: First, that the symptoms of anaphylaxis and certain allergic diseases are produced by histamine, which is released during the antigen-antibody reaction; and secondly, that the most outstanding property of Pyribenzamine and related substances is their antihistaminic power (Mayer, Huttrer and Scholz,[1] Mayer[2]).

Many clinical observations on the activity of Pyribenzamine and similar substances in various forms of allergic manifestations appear to sustain this explanation. The effectiveness of antihistaminic therapy depends in a large measure upon the type of allergy against which it is used, and antihistaminics are most effective in conditions in which histamine release is suggested by experimental findings, namely in anaphylaxis, serum disease, urticaria or hay fever. On the other hand, if the activity of these substances is due solely to the inhibition of histamine, then they should be ineffective in allergic dermatitis or eczema, since none of the clinical symptoms of contact dermatitis or eczema appear to have any resemblance to the known histamine symptoms. To date, all attempts to produce these forms of skin reactions by the administration of histamine have been unsuccessful.

But in spite of these facts, there are numerous clinical reports citing the effectiveness of the various antihistaminics in contact dermatitis and atopic eczema (Feinberg,[3] Chobot[4]), and one of us has shown that Pyribenzamine exerts a considerable prophylactic activity in experimental contact dermatitis of guinea pigs (Mayer[5,6]). Since the effectiveness in these epidermal sensitizations is difficult to explain on the basis of inhibition of histamine, it appears more likely that antihistaminic substances act in these cases by virtue of a *pharmacodynamic property different from their action upon proven histamine effects. All antihistaminic substances developed thus far possess, in addition to their antihistaminic power, various degrees of antispasmodic and local anesthetic activities. It is possible that the curative effect in dermatitis and eczema may* be explained as being due to the local anesthetic property, although several observations contradict this view.

Recent investigations on the mechanism of epidermal inflammations due to the action of chemical or physical influences have pointed to the importance of certain amines, other than histamine, and of substances such as leukotaxine, pyrexin and necrosin (Menkin[7]). Although the significance of these substances

1 Mayer, R. L., Huttrer, G. P., and Scholz, C. R., *Science*, 1945, **102**, 93.

2 Mayer, R. L., *J. Allergy*, 1946, **17**, 153.

3 Feinberg, S. M., *J. A. M. A.*, 1946, **132**, 702.

4 Chobot, R., *J. Allergy*, 1947, **18**, 76.

5 Mayer, R. L., *J. Invest. Dermat.*, 1947, **8**, 67.

6 Mayer, R. L., *Ann. Allergy*, 1947, **5**, 113.

7 Menkin, V., *Arch. Path.*, 1946, **41**, 376.

Tail   Head

FIG. 1.

Rat No. 32, right side, before Pyribenzamine. Tail: India ink plus saline. Head: India ink plus 1% hyaluronidase.

Note: Dark spot upper center is due to the subsequent injection of Pyribenzamine.

is somewhat uncertain. Cullumbine[8] has investigated the influence of antihistaminic substances on the action of leukotaxine; he could not detect any anti-inflammatory activity.

On the other hand, various inflammatory processes produced by strongly invasive micro-organisms have been associated with the release, from these organisms, of a specific enzyme, namely hyaluronidase (Duran-Reynals[9]). So far, very little is known concerning the role which this enzyme plays in non-bacterial inflammations of the skin. Since relatively large concentrations of hyaluronidase occur in the normal skin, this may indicate a more general function. (Meyer et al.,[10] Meyer[11]) Certain of the observations described below indicate that hyaluronidase may be associated with certain phases of skin inflammations, especially of allergic non-bacterial nature.

We have directed our investigations in this direction and have studied the influence of Pyribenzamine and antistine upon the activity of hyaluronidase both as a spreading factor and as a possible factor responsible for allergic inflammation of the skin.

Experimental. A. Spreading of India ink in rats. The effect of Pyribenzamine and antistine upon the diffusion of India ink in the skin of albino rats in the presence and absence of hyaluronidase was investigated according to the method described by Duran-Reynals[8] and Cahen and Granier.[12] One hundred and twenty male and female rats, each

8 Cullumbine, H., *Nature*, 1947, 159, 841.

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11 Meyer, K., *Physiol. Rev.*, 1947, 27, 335.

12 Cahen, R. L., and Granier, M., *Yale J. Biol. and Med.*, 1944, 16, 257.

Tail   Head*
FIG. 2.
Rat No. 32, left side, after 37.5 mg of Pyribenzamine. Tail: India ink plus saline.
Head: India ink plus 1% hyaluronidase.
* This photograph has purposely been reversed in order to permit a comparison of
Fig. 1 and 2.

weighing between 120 and 220 g, were treated simultaneously at 2 sites on the right flank; one site being injected intradermally with a mixture of 0.05 cc of Higgins India ink and 0.10 cc physiological saline, and the other site with a mixture of 0.05 cc of ink and 0.10 cc 1% hyaluronidase solution. The hyaluronidase preparation* used in these experiments contained 150 turbidity-reducing units per milligram. Twenty hours later the areas were outlined in ink, traced on cellophane sheets, and measured with a planimeter.

Having thus determined the *in vivo* activity of the hyaluronidase preparation, the animals were subcutaneously injected on the right side 24 hours after the India ink injections with a solution of Pyribenzamine or antistine in doses of 15, 37.5 and 75 mg per kg of body

weight respectively; the highest dose representing ⅓ to ⅕ of the LD[50] for rats (Mayer *et al.*[13]).

Twenty minutes after the administration of the antihistaminic, the intradermal injections of India ink plus saline, and India ink plus hyaluronidase were repeated on the corresponding areas of the left flank. The areas of spreading were measured as outlined above.

The results of this India ink test, given in Table I, show that the antihistaminic substances Pyribenzamine and antistine strongly reduced both the extent of the normal spreading of injected India ink, as well as the spread produced in the presence of hyaluronidase. Doses of 15, 37.5 and 75 mg/kg of Pyribenzamine restricted the area of spreading pro-

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* We are indebted to Dr. E. Schwenk, of the Schering Corp., who kindly supplied the material.

[13] Mayer, R. L., Hays, H. W., Brousseau, D., Mathieson, D., Rennick, B., and Yonkman, F. F., *J. Lab. Clin. Med.*, 1946, **31**, 749.

duced by hyaluronidase by 6%, 21% and 44% respectively over that of the control areas. Similar doses of antistine restricted the spreading by 6%, 20% and 51% respectively. Fig. 2 shows the decrease of the hyaluronidase spreading effect after injection of 37.5 mg/kg Pyribenzamine, in contrast to the hyaluronidase effect on the same animal before Pyribenzamine treatment, as shown in Fig. 1.

Morphine sulfate when tested according to the method of Cahen and Granier[12] in doses of 25 and 32 mg/kg, restricted the increase of spreading by 46% and 47% respectively.

B. *Effect of Antihistaminics Upon the Action of Hyaluronidase in Experimental Sensitizations.* 1. *Action of Hyaluronidase upon Allergic Inflammation.* We sensitized 18 guinea pigs to paraphenylenediamine by treatment with 10% paraphenylenediamine ointment according to a method previously described (Mayer[14]) and challenged them 3 weeks later with an intradermal injection of 0.10 ml of 0.75% paraphenylenediamine solution.

The paraphenylenediamine used for the challenge was freshly recrystalized and solutions prepared in the absence of oxygen, in order to minimize the formation of oxidation products which could destroy the hyaluronidase. Since the solutions were slightly tinted in spite of these precautions, 10% horse serum was added immediately before the injections as a further precaution, the serum proteins combining with and simultaneously reducing the small amount of oxidation product present. Although it is known that certain sera inhibit the action of hyaluronidase (McClean[15]), Hechter and Scully[16] have recently shown that serum interferes only after incubation; in our experiments no significant diminution of the spreading effect took place.

The challenge injections with antigen alone and with antigen-hyaluronidase mixtures were performed in the same manner as described above for the India ink experiments in rats. The areas of inflammation and infiltration.

14 Mayer, R. L., *Arch. f. Dermatol. u. Syph.*, 1931, 163, 223.

15 McClean, D., *J. Path. and Bact.*, 1942, 54, 284.

16 Hechter, O., and Scully, E. L., *J. Exp. Med.*, 1947, 86, 19.

TABLE I.
Effect of Pyribenzamine and Antistine upon the Spreading of India Ink in the Presence and Absence of Hyaluronidase.
20 rats tested in each experiment.

Exp.	Dosage (mg/kg)	India ink alone %	India ink plus hyaluronidase %
	Pyribenzamine		
1	15	$14.05 \pm 4.21^*$	6.25 ± 6.39
2	37.5	18.70 ± 5.20	21.10 ± 3.77
3	75	47.85 ± 2.53	43.95 ± 3.73
	Antistine		
4	15	13.65 ± 5.73	11.10 ± 4.77
5	37.5	31.75 ± 5.22	20.00 ± 4.23
6	75	28.10 ± 4.99	37.00 ± 3.22

* $\pm$ values represent probable error of the mean.

varying from 6 mm to 25 mm in diameter, were measured and at the same time, the intensity of infiltration and redness noted by symbols as indicated in Table II.

The results obtained indicate that hyaluronidase increases the spread of the allergic epidermal reaction in sensitized guinea pigs, as it increases the spread of India ink in rats. The average challenge reaction in sensitized animals measured 2.3 mm^2 when the antigen was injected alone, and 16.9 mm^2 when it was combined with hyaluronidase. Simultaneously, there was a marked increase in the *intensity* of the allergic inflammation which was even more significant than the increase in the size of the reaction. The intensity increased from a $+$ reaction to a $+++$ or $++++$ reaction in the presence of hyaluronidase, in spite of the fact that the greater spread, as proven by the increased area of inflammation, had resulted in a marked dilution of the antigen concentration at the site of reaction.

2. *Influence of Pyribenzamine Upon the Hyaluronidase Effect in Allergic Inflammations. a. Sensitization to paraphenylenediamine.* Twenty-four hours after the spreading test was performed these animals received 15 mg/kg Pyribenzamine subcutaneously on the same flank. Twenty minutes later they were retested on the opposite flank with paraphenylenediamine and a paraphenylenediamine-hyaluronidase horse serum mixture in the same manner. Since the challenge reaction is in this case an epidermal inflammation of the contact dermatitis or tuberculin type, which

TABLE II.

Influence of Hyaluronidase on the Challenge Reaction in Experimental Epidermal Sensitizations to Paraphenylenediamine (PP) in Guinea Pigs.

| | | Average size and intensity of challenge reaction | | | |
| | | Without hyaluronidase | | With hyaluronidase | |
	No. of animals	Size in mm²	Intensity of inflammation	Size in mm²	Intensity of inflammation
Controls	4	0.317 ± 0.124	(+)	1.60 ± 0.14	+
Sensitized animals	18	2.3 ± 0.74	+	16.9 ± 0.52	+++ to ++++

Signs: (+) to ++++ represent varying degrees of inflammation as measured by the intensity of redness and degree of infiltration.

TABLE III.

Influence of Pyribenzamine upon the Allergic Reaction in Guinea Pigs to Paraphenylenediamine in Presence and Absence of Hyaluronidase.

| | | Average size and intensity of local challenge reaction | | | |
| | | Without hyaluronidase | | With hyaluronidase | |
	No. of animals	Size in mm²	Intensity of inflammation	Size in mm²	Intensity of inflammation
Controls	4	0	0	0.75 ± 0.48	0
Sensitized animals	18	0.27 ± 0.14	0	2.7 ± 0.54	(+)

15 mg per kg body weight of Pyribenzamine was given twice subcutaneously, namely, 15 to 20 minutes before and 4½ hours after the challenge injection of the antigen.

develops gradually, reaching a maximum only after 12 to 16 hours, an additional dose of Pyribenzamine (15 mg/kg) was injected 4½ hours later.

The results of this experiment are given in Table III. The area of the challenge reaction to the antigen alone this time was found to be to an average size of 0.27 mm², against 2.3 mm² before the Pyribenzamine injection. This reaction became even smaller than the unspecific, non-allergic reaction to the paraphenylenediamine injection in non-sensitized control animals. The average size of the challenge reaction produced in the presence of hyaluronidase was reduced from 16.9 mm² to 2.7 mm², a value close to that observed in the animals having received antigen alone (Table III). Thus the pretreatment with Pyribenzamine almost completely suppressed the allergic reaction regardless of whether the antigen was injected with or without hyaluronidase.

b. Sensitization to 2,4-dinitrochlorobenzene. In a previous study it was shown that antihistaminic substances prevent or diminish the epidermal manifestations of experimental sensitizations of guinea pigs to 2,4-dinitrochloro-

benzene (Mayer[14]). This type of sensitization differs from that produced by paraphenylenediamine in the clinical aspect of the challenge reaction, as well as in the histological changes produced. It was therefore of interest to investigate whether the challenge reaction in sensitizations to this nitro compound was also reinforced by hyaluronidase, as was the case in sensitizations to the diamine.

Ten guinea pigs were sensitized to 2,4-dinitrochlorobenzene by intradermal injection of 0.02 mg of antigen, and after 27 days all animals had become strongly allergic to the substance. They were then challenged intradermally with 0.1 ml containing 0.02 mg of antigen alone and with a mixture of 0.02 mg of antigen and 0.5 mg of hyaluronidase in a similar manner, as described for paraphenylenediamine. However, in this case no horse serum was added to the antigen-hyaluronidase mixture, since, according to Landsteiner[17] 2,4-dinitrochlorobenzene does not react with protein at room temperature.

In this case, however, hyaluronidase neither increased the size nor the intensity of the

17 Landsteiner, K., *The Specificity of Serological Reactions*, Cambridge, Mass., 1945.

challenge reaction. It is not known yet whether this negative result is due to a destruction of hyaluronidase, or to a blocking of its action by the nitro compound, or whether this type of epidermal sensitization does not intrinsically respond to hyaluronidase.

C. Role of the Local Anesthetic Activity of Antihistaminics. Pyribenzamine and the other antihistaminic substances of similar chemical constitution have local anesthetic activity equal to or superior to that of procaine. It was therefore of interest to determine whether a local anesthetic substance with low antihistaminic activity, such as procaine, influenced the spreading effect of hyaluronidase in the same way as Pyribenzamine.

Twenty rats were tested as outlined above and the influence of procaine upon the spread of India ink investigated at two different levels, namely 75 mg procaine per kg of body weight, which corresponded to the highest doses of Pyribenzamine used in the earlier experiments and 225 mg per kg of body weight, representing a 3-fold increase in the local anesthetic action.

Seventy-five mg/kg of procaine had no significant influence upon the spread of India ink either in the absence or presence of hyaluronidase. With 225 mg per kg procaine, the spread of India ink was decreased by 11% in the absence of hyaluronidase, and 18% in experiments performed in the presence of hyaluronidase. The activity of this high concentration of procaine could be due to an unspecific cell injury, since it was highly irritating locally and produced deep necrosis.

According to these results, procaine has a slight influence upon the normal spread of India ink, as well as on the spreading effect of hyaluronidase. It is, however, much less active than Pyribenzamine or antistine in this respect.

Discussion. The present experimentation was performed to determine (a) whether hyaluronidase plays a role in allergic, nonbacterial inflammation of the skin and (b) whether antihistaminic substances have an anti-hyaluronidase activity.

The results indicate that hyaluronidase markedly influences the epidermal challenge reaction in guinea pigs sensitized to paraphenylenediamine, its presence increasing the size and intensity of inflammation. Furthermore, the fact that antihistaminic substances markedly decrease the effect of hyaluronidase upon the spread of India ink and upon the allergic reaction to paraphenylenediamine constitutes, we believe, reasonable indication that hyaluronidase does play a role in certain nonbacterial, especially allergic inflammation, as it does in certain bacterial processes. The direct proof for this theory, however, is still lacking since it has not been shown that hyaluronidase is liberated during the allergic inflammation of the skin (and other organs) and that, if its amount is increased during this inflammation, it is etiologically related with some phase of the allergic reaction. So far, certain technical difficulties have been encountered in the interpretation of experiments performed to elucidate this point.

The present investigation has furthermore disclosed that antihistaminic substances such as Pyribenzamine and antistine possess, in addition to their antihistaminic activity, other properties which may permit action in various manifestations thus far not associated with histamine-release. The strong anti-hyaluronidase effect, which results in a reduction of the size as well as the intensity of an allergic inflammation, may explain the activity of Pyribenzamine and similar substances in epidermal sensitizations such as eczema or contact dermatitis. It might also explain the effect antihistaminics seem to exert in certain rheumatic manifestations, where, according to Guerra, salicylates act by a similar mechanism.

It is not yet known whether this anti-hyaluronidase effect is a specific pharmacologic property of Pyribenzamine and antistine, comparable, for instance, to their specific antihistaminic power. It is quite possible that the effect is the result of a nonspecific process. On the other hand, the local anesthetic power may, to a certain extent, be associated with this activity—a question which could be decided by the use of antihistaminic substances devoid of local anesthetic power.

Conclusions. Experiments are presented which show that (a) hyaluronidase increases the intensity of allergic skin inflammation of

TABLE IV.

Influence of Procaine upon the Spreading of India Ink in the Presence and Absence of Hyaluronidase. 10 rats tested in each experiment.

Exp.	Dosage (mg/kg)	Average size of spreading		Reduction of spreading area	
		Without hyaluronidase	With hyaluronidase	Without hyaluronidase	With hyaluronidase
				%	%
1	0	5.92	8.9		
	75	5.8	8.4	3.8 ± 6.4	5.3 ± 4.5
2	0	6.1	8.8		
	225	5.3	7.0	11.1 ± 3.4	18.1 ± 3.3

the epidermal type and (b) that antihistaminic substances such as Pyribenzamine and antistine counteract the spreading effect of India ink and hyaluronidase, as well as the latter's effect upon the allergic skin inflammation. The significance of these findings for the activity of antihistaminics in epidermal sensitizations is discussed.

16102

Isolation and Properties of Snake Erythrocyte Nuclei.

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The separation of nuclei from avian erythrocytes has been attempted by Warburg and by Miyake using the freezing and thawing technique.[1,2] However, the nuclei obtained by this method are not well separated and usually agglutinate in a mass. Laskowski was able to prepare a stable suspension of free nuclei of chicken blood using lysolecithin in neutral phosphate buffer solution for the hemolysis with subsequent washing of the nuclei with isotonic saline.[3] The poison glands of bees were ground with the lecithin emulsion which was then incubated for 24 hours at 37°C and finally filtered through a Berkefeld filter. The lysolecithin so prepared was added to the blood and after the hemolysis the nuclei were separated by centrifugation. This technique yields good preparations but the lysolecithin is not easily obtained under standard conditions. The procurement of the bee poison and the time required are disadvantageous conditions when large amounts of blood are used for chemical analysis.

The well known hemolytic action of tyrothricin, an antibiotic obtained from *Bacillus brevis* and commercially available,* has been used in our experiments with very satisfactory results. Snake erythrocytes after rapid hemolysis liberate the nuclei which are then washed several times in 0.9% saline and 5% citric acid solution. Microscopic examination of the nuclei suspension shows that they are not agglutinated and are almost free from the cytoplasm (Fig. 1). They are easily stained with methylene blue and Giemsa. The nuclei in suspension appear microscopically smaller and more compact than in the intact erythrocytes. This observation was also reported by Laskowski for chicken blood preparations.

1 Warburg, O., *Z. physiol. Chemie*, 1910, **70**, 413.

2 Miyake, M., *Keijo J. Med.*, 1933, **4**, 247.

3 Laskowski, M., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 354.

* Tyrothricin 2% solution, Parke, Davis & Co.
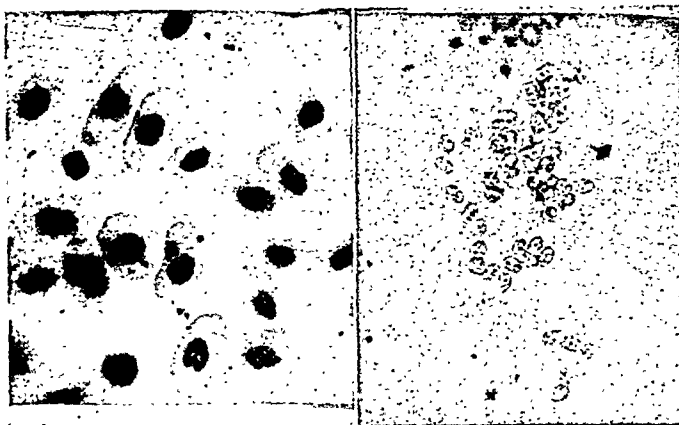
FIG. 1.
Left: Nuclei of the erythrocytes of the snake *Eudryas bifossatus*
obtained with the tyrothricin hemolytic technique. Right: Blood
of the same snake stained with Giemsa. × 810.

Fig. 1 shows a microphotograph of the nuclei of erythrocytes of snake blood suspended in 5% citric acid in comparison with the nuclei in the intact erythrocytes stained with Giemsa.

Method. The blood of 2 poisonous and 4 non-poisonous snakes was studied (Table I). It was collected from decapitated snakes directly into large test tubes containing 0.3% potassium oxalate. The amount obtainable (usually 25 to 40 ml) depended upon the size of the reptile. After centrifugation the plasma and leucocytes were pipetted off and discarded. The erythrocytes were washed several times (5 to 7) with 0.9% NaCl solution, until the washing was water clear. The densely packed cell layer was mixed with saline in a stoppered flask to obtain a 5% suspension. To 40 ml of the erythrocyte suspension 1 ml of the tyrothricin solution (20 mg/ml)† was added. The suspension was mixed well immediately by inversion of the flask. After 10 minutes the hemolyzed blood was centrifuged and the whitish mass of nuclei washed twice with saline, once with 5% citric acid, and finally suspended in the

citric acid solution. For chemical analysis the citric acid treatment was omitted. The suspension of the nuclei was dehydrated with 95% alcohol in a centrifuge tube and the alcohol was evaporated under low pressure until a fine white powder was obtained. The powder was left over night in a vacuum desiccator and stored in the refrigerator. By this technique 74 mg could be prepared from 5 ml of washed snake erythrocytes. The same technique was applied successfully to the blood of fowls.

Results. Applying the saponin method of Dounce and Lan[4] to the erythrocytes of some snakes we obtained good preparations, but the nuclei still showed a layer of stroma. The authors cited demonstrated that the nuclei from chicken erythrocytes, which are somewhat yellow in color, contain a xanthophyll pigment and probably a flavin. Since the plasma of some snakes *(Bothrops, Eudryas)* is very rich in riboflavin, as formerly reported by us[5] it would be of interest to know if the nuclei of the erythrocytes also contain flavins. Using both tyrothricin and saponin techniques, we were unable to detect any flavin or carotenoid pigment in any sample exam-

4 Dounce, A. L., and Lan, T. H., *Science*, 1943, 97, 584.

5 Villela, G. G., and Prado, J. L., *J. Biol. Chem.*, 1945, 157, 693.

TABLE I.
Desoxyribonucleic Acid Content of Nuclei of the Snake Erythrocytes.

Common names	Scientific names	No. of samples	Mg of desoxyribo-nuclei acid in 100 mg dry nuclei
Non poisonous snakes:			
Limpa-campo	*Eudryas bifossatus*	3	64
Giboia	*Boa sp.*	1	62
Caninana	*Spillotis pullatus*	2	31
Boipeva	*Xenodon merrimii*	1	44
Poisonous snakes:			
Rattlesnake (Cascavel)	*Crotalus terrificus*	3	59
Jararaca	*Brothrops jararaca*	4	56

ined. The ether extract and the remaining nuclei mass showed only a slight white fluorescence. Thiamin and nicotinamide assays on the dry nuclei showed values averaging 80 and 122 γ/g respectively (4 samples analyzed). The microbiological methods of Sarett and Cheldelin[6] for thiamin and of Krehl, Strong and Elvehjem[7] for nicotinamide were employed. The nuclei assayed were only saline-washed. Citric acid-treated nuclei are not suitable for analysis by these methods.

The desoxyribonucleic acid content of the nuclei was determined by use of the Dische reaction, as adapted by Dounce to permit spectrophotometric measurement of the blue color.[8] In a test tube 2 mg of the alcohol-dryed nuclei were suspended in 0.5 ml of distilled water and 1.0 ml of the Dische reagent. The tubes were immersed in a boiling water bath and 8.5 ml of the blank reagent (sulfuric-acetic acid mixture) was added. The slight turbidity appearing 1 to 2 minutes later was easily removed by the addition of 0.1 to 0.2 g of celite (Hyflo-supercel) and subsequent filtration or centrifugation. The clear blue color was measured in a Lumetron photocolorimeter using a 530 mμ filter. The blank value was subtracted from the unknown. To the tube containing the blank

6 Sarett, H. P., and Cheldelin, V. H., *J. Biol. Chem.*, 1944, **155**, 153.

7 Krehl, W. A., Strong, F. M., and Elvehjem, C. A., *Ind. and Eng. Chem., Anal. Ed.*, 1943, **15**, 471.

8 Dounce, A. L., *J. Biol. Chem.*, 1943, **151**, 231.

were added 1.0 ml of the Dische reagent and 7.5 ml of the blank reagent (sulfuric-acetic mixture) so that the total volume in the blank and unknown tubes was 10 ml. The color was stable for only 20 minutes and, therefore, all the operations had to be run in a short time. The values, referred to as desoxyribonucleic acid, were calculated from a calibrated curve made with pure sodium desoxyribonucleate prepared from normal liver nuclei according to a modified Hammarsten method and tested both spectrophotometrically and chemically (N : P ratio = 2.34%). The averages obtained for the nuclei of the different snake erythrocytes are shown in Table I.†

The percentage of desoxyribonucleic acid found in the nuclei of snakes showed higher values than those reported by Dounce for the nuclei of chicken erythrocytes and of rat liver (about 45%).[4,8] The total lipid content was determined only for the nuclei of the snakes *Spillotis pullatus* and *Eudryas bifossatus* and averaged 12.7%. Dounce and Lan reported for the chicken the total lipid content of about 14%.[4]

Summary. A new technique for the isolation of the snake erythrocyte nuclei using tyrothricin as an hemolytic agent is described. Some properties and chemical data are reported. The desoxyribonucleic acid content of the erythrocyte nuclei for the 6 kinds of snakes studied was higher than that reported for chicken erythrocytes and liver rat nuclei.

16103

Attempted Infection of the Hen and Man with the Sporozoites of *Plasmodium cathemerium 3H2.*[*]

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Huff and Coulston[1] reported their inability to find cryptozoites in canaries inoculated with the sporozoites of *Plasmodium gallinaceum*, a parasite to which the chick is quite susceptible, and also their failure to establish infections in canaries by the inoculation of blood from chickens infected with this organism. The present communication deals with a reversed experiment, *i.e.*, the attempt to infect the chicken with both the sporozoites and the trophozoites of a parasite that is highly virulent in canaries. The organism used in these trials was the *P. cathemerium 3H2* that has been continuously maintained through mosquito-canary-mosquito passages in this laboratory since February, 1937. In addition there will be placed on record here an attempt to infect man with this organism, which I believe has not previously been reported.

First Trial. Using heparin as anticoagulant. 5 cc of blood was drawn by cardiac puncture from each of 3 full-grown white Leghorn hens and the plasma obtained after pooling the samples. Then 100 *Culex pipiens* mosquitoes that had fed two weeks previously on canaries whose blood contained numerous gametocytes of *P. cathemerium 3H2* were stunned in a flask, ground in 2.5 cc of the plasma, and washed through 3 layers of gauze with another 2.0 cc of plasma; the volume of the filtrate was then made up to 5 cc with additional plasma. Of this filtrate, 0.1 cc (representing the sporozoites from 2 mosquitoes) was immediately injected intramuscularly into each of 4 clean canaries and 0.3

cc (6 mosquitoes) intramuscularly into each of 4 white Leghorn hens. Plasmodia were found in the peripheral blood of the canaries on the 7th, 7th, 8th and 9th days, respectively, but the hens remained negative through 17 days. On the 18th day the blood drawn by cardiac puncture from each of the 4 hens was pooled and 0.1 cc injected intramuscularly into each of 4 clean canaries; the blood of these birds remained negative through 16 days but all 4 birds subsequently became infected when challenged by the bites of infected mosquitoes.

Second Trial. Hen blood was obtained as in the preceding trial but it was allowed to stand after cooling at room temperature for one hour before centrifuging to obtain the plasma. Thereafter all steps were as in the preceding trial and the results were precisely the same.

Third Trial. One hundred infected mosquitoes were ground in 1.0 cc of Locke's solution for 30 seconds and the filtrate made up to 1.0 cc with Locke's solution; 0.2 cc of this filtrate was then diluted to 2.0 cc with Locke's solution and 2 hens were each injected intramuscularly with 0.5 cc (5 mosquitoes) of this mixture and 2 canaries with 0.1 cc (1 mosquito). Again the same results were obtained.

Fourth and Fifth Trials. These were repetitions of the immediately preceding trial, with the same results.

Sixth Trial. In this trial the mosquito-Locke filtrate was added to whole canary blood and rotated in the incubator at 41.5°C for 2½ hours before injecting into the hens and canaries. The results were the same as in all the preceding trials.

Seventh Trial. On May 8, 1947, D.P. and H.B. were bitten on the forearm by mosquitoes infected with *P. cathemerium 3H2* in the

usual way on April 23; D.P. received 11 bites and H.B. 17 bites. The first peripheral blood smears were examined on May 14 and thereafter on May 16, 20, 23, 27 and 30; all the smears were negative. On June 19 (6 weeks after the infective bites), 5.0 cc of blood were drawn from each of the subjects, pooled, and 0.1 cc at once injected into each of 5 canaries. The blood of these canaries remained negative through 16 days of smearing, but it subsequently became positive after the usual incubation period when the birds were challenged by the bites of infected mosquitoes.

Summary. The sporozoites of *P. cathemerium 3H2*, an organism causing severe infections in canaries, were injected intramuscularly in large numbers into full-grown barnyard hens. In the respective trials the injectum consisted of (a) the gauze filtrate of infected mosquitoes ground in fresh hen plasma; (b) the gauze filtrate of infected mosquitoes ground in hen plasma obtained after allowing the drawn blood to stand for one hour at room temperature; (c) the gauze filtrate of infected mosquitoes ground in Locke's solution; (d) whole canary blood to which was added the gauze filtrate of infected mosquitoes ground in Locke's solution, the mixture being incubated at the temperature of hen blood for 2½ hours before using for injection. None of the injected hens in any of the trials became infected and all of the canaries injected as controls did become infected.

Two human volunteers were bitten, respectively, by 11 and 17 mosquitoes infected with *P. cathemerium 3H2;* neither of the individuals became infected so far as could be determined by a study of peripheral blood smears and subinoculation of blood into canaries.

16104

Therapeutic Efficacy in Experimental Syphilis of Eight Daily Injections of Penicillin in Beeswax-Peanut Oil.*

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Many methods have been developed to prolong the therapeutic activity of penicillin preparations, but the most effective of these appears to be the use of calcium penicillin in beeswax and peanut oil.[1] Romansky[2] has demonstrated that therapeutic penicillin levels are maintained in man for approximately 24 hours following the single injection of 300,000 units of calcium penicillin in 4.8% beeswax by weight in peanut oil, contained in 1 cc. From a practical standpoint the use of this preparation permits active therapy to be carried out on an ambulatory basis. The advantages of such a procedure in the treatment of syphilis are obvious.

The present report summarizes the results of an experiment designed to assay the effectiveness of calcium penicillin in beeswax and peanut oil in the treatment of experimental rabbit syphilis, when the calculated total dose is administered in 8 daily injections.

Material and Methods. Male rabbits weighing from 2.5 to 4.2 kilograms were utilized. They were housed in individual cages and maintained on commercial pellets

augmented by a free supply of water. The environmental temperature was stabilized through air-conditioning at a level never exceeding 75°F. Bilateral intratesticular inoculations were carried out with saline emulsions of active syphilitic orchitic material from routine passage animals infected with the Nichols strain of *Tr. pallidum*. The inoculated animals were then observed for the development of orchitis, and darkfield examinations were performed when clinically indicated. Only darkfield positive animals were included in the treatment groups.

Treatment was begun 6 weeks following inoculation. All animals were weighed on the day prior to the beginning of treatment and the total dosage for each animal was calculated on the basis of these weights. The total amount of drug was intramuscularly injected in 8 equally divided daily injections, through the shaved skin of the hind quarters. The preparation consisted of calcium penicillin in 4.8% beeswax (by weight) in peanut oil, prepared and kindly supplied to us by Dr. Harry Eagle. Three dilutions were utilized. The first 2 were labelled "Abbott-Eagle Lot No. 2" and contained 125 units and 1,000 units of calcium penicillin per cc respectively. The third contained 8,000 units of calcium penicillin per cc and was labelled "Squibb." The Squibb preparation was employed only in the highest dosage group, *i.e.,* 16,000 units per kilo. The Abbott material was used in all other dosages.

The animals were observed for 4 months following the completion of treatment. During this period frequent clinical examinations were conducted and all suspicious lesions were examined by darkfield. If positive, the animal was considered to be a treatment failure and discarded. At the end of 4 months all surviving animals were sacrificed, and the aseptically removed popliteal lymph nodes from each animal were emulsified with equal parts of saline and rabbit serum. The resulting emulsion prepared from each rabbit was then inoculated into each testicle of 2 normal animals. These were held for 3 months and observed by frequent clinical examinations. Suspicious lesions were dark-

fielded, and when this examination was positive, the parent animal from whom the inoculated popliteal lymph nodes were secured, was deemed to be a treatment failure. At the end of 3 months the surviving transfer animals were sacrificed and the sites of inoculation examined by darkfield for the presence of treponemes. If these were found, the parent animal was considered to be a treatment failure. If this final examination was negative, the parent animal was listed as a treatment success.

There were 7 groups of treated animals. Treatment varied between groups from a total dosage of 250 units to 16,000 units per kilo of body weight. Two different experiments were conducted. The first series of animals was inoculated on February 14, 1946, and the second on September 6, 1946. The results in these 2 series were not significantly different. They were therefore combined and are reported here as a single experiment.

Results. The final results are summarized in the accompanying table. It will be noted that all of 4 rabbits receiving a total dose of 250 units per kilo were treatment failures, while all of 14 animals receiving 8,000 or more units per kilo total dose were treatment successes. There is a fairly uniform increase in the ratio of successes with increasing dosage. The CD_{50} or the amount of drug required to cure 50% of the animals, when calculated according to the Reed-Muench[3] formula, was 1,070 units per kilo.

Discussion. Employing the identical method of assay, Eagle, Magnuson and Fleischman[4] have reported a CD_{50} of 1,600 units per kilo when sodium penicillin in aqueous solution was employed, the total dosage having been given in 5 injections per day for 4 days. Penicillin G, injected every 4 hours for 24 treatments has been found[5] to result in a CD_{50} of 1,450 and 1,600 units per kilo.

[3] Reed, L. J., and Muench, W., *Am. J. Hyg.,* 1938, 27, 493.

[4] Eagle, H., Magnuson, H. J., and Fleischman, R., *Bull. Johns Hopkins Hosp.,* 1946, 79, 168.

[5] Arnold, R. C., Boak, R. A., Carpenter, C. M., Chesney, A. M., Fleming, W. L., Gueft, B., Mahoney, J. F., and Rosahn, P. D., *Am. J. Syph., Gon. and Ven. Dis.,* in press.

TABLE I.

Results of Treatment of Experimental Rabbit Syphilis with calcium penicillin in Beeswax and Peanut Oil. Total calculated dose given in 8 daily injections.

No. of animals	Total dosage units/kg	Failed	Cured	Total Failed*	Total Cured*	% cured*
4	250	4	0	16	0	0
7	500	5	2	12	2	14
9	1000	5	4	7	6	46
9	2000	1	8	2	14	87
9	4000	1	8	1	22	95
9	8000	0	9	0	31	100
5	16000	0	5	0	36	100

* After Reed and Muench.[3]

Our results with 8 daily injections of calcium penicillin in beeswax and peanut oil were essentially no different from these reported for sodium penicillin and for penicillin G.

Fleming[6] assayed a commercial preparation of penicillin in beeswax and peanut oil in the same manner as that reported herein, but his schedule employed 16 daily injections instead of 8. The CD_{50} in his experiment was 1,400 units per kilo. This value is not significantly different from our own findings of 1,070 units per kilo, when the two results are compared by the method of Behrens[7] (difference $= 1.34 \pm 1.07$, t $= 1.25$). It is apparent that the total curative dose (CD_{50}) is not significantly altered by increasing the number of daily injections from 8 to 16. This observation may have important clinical implications in the treatment of human syphilis.

Summary. Calcium penicillin in beeswax and peanut oil was employed to treat groups of rabbits infected with *Tr. pallidum*. The calculated total dose based on body weight was administered in 8 daily injections. A CD_{50} of 1,070 units per kilo of body weight was observed. This value does not differ significantly from a CD_{50} of 1,400 units per kilo reported by Fleming following administration of a similar preparation in 16 equal daily doses. It was concluded that when calcium penicillin in beeswax-peanut oil is employed in the treatment of rabbit syphilis, increasing the number of daily injections from 8 to 16 does not alter the total curative dose (CD_{50}).

6 Fleming, W. L., presented at a symposium, *Recent Advances in the Investigation of Venereal Disease*, Washington, D.C., Apr. 17, 1947.

7 Behrens, B., quoted by Gaddum, J. H., Reports on Biological Standards III. Methods of Biological Assay Depending on a Quantal Response, *Medical Research Council, London*, Special Report Series, No. 183, 1933, p. 26.

16105

A Search for *Treponema pallidum* in the Lymph Nodes of the Syphilitic Mouse.[*]

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In the course of an investigation of experimental mouse syphilis, it was found important to be certain that all the mice inoculated with syphilis actually became infected and harbored the *Treponema pallidum*. As Kolle and Schlossberger[1] first showed in 1926, symptoms do not appear despite an infection which persists throughout the life of the mouse. One method of ascertaining the presence of infection is to subinoculate mouse tissues into rabbit testicle. A darkfield positive syphiloma will usually develop after 20 to 80 days if the inoculated tissues contained viable *Treponema pallidum* in amounts sufficient to infect. This is a cumbersome method and the question was raised whether the treponemes might be easily found by darkfield search of excised node tissue, as the nodes are known to be highly virulent. Also, Jahnel and Prigge[2] demonstrated the presence of *Treponema pallidum* in the lymph nodes of the infected mouse by using silver impregnation methods. Levaditi and Rousset-Chabaud[3] examined skin biopsies of syphilitic mice impregnated by Stroesco's silver method, to exclude noninfected mice from therapy trials. However, this method uses fixed tissue and the *Treponema pallidum* may be confused with the *Spirillum morsus muris* or other spirochetes often found in the asymptomatic mice. Two observers have reported finding the *Treponema* in nodes by darkfield examination.

Kato[4] was the first in 1931 and Bessemans and his co-workers Van Haelst and De Moor have repeatedly found and counted the treponemes in mouse lymph nodes[5,6] after extremely laborious searches.

Kato reported finding the *Treponema pallidum* frequently in the iliac nodes of mice inoculated intrascrotally one to 40 days previously with rabbit chancre tissue. His method was to divide the node into 5 fragments, emulsify each fragment in a drop of normal saline, and search each fragment with the darkfield microscope, examining 50 fields per fragment, or a total of 250 fields per node. His results are given in Table I.

Van Haelst[6] found it necessary to examine from 500 to 3,000 darkfields of node tissue emulsified in such a way as to allow enumeration of the treponemes seen. He found that almost all nodes examined, from 56 to 425 days after infection, contained visible characteristic treponemes. Karrenberg[7] attempted to repeat Kato's findings and reported failure in a very complete review of mouse syphilis. He mentioned seeing "spirochetoid" movements but no treponemes. The apparent ease with which Kato found treponemes led to the present attempt to use his method. It was hoped that examination of an excised mouse lymph node would suffice to establish the presence of syphilitic infection in the mouse, and that rabbit inoculations of control mice

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[1] Kolle, W., and Schlossberger, H., *Deutsche Med. Wchnschr.*, 1926, 52, 1245.

[2] Jahnel, F., and Prigge, R., *Deutsche Med. Wchnschr.*, 1929, 55, 694.

[3] Levaditi, C., and Rousset-Chabaud, D., *Bull. Acad. de Méd. Paris*, 1941, 124, 176.

[4] Kato, N., *Bull. Soc. Japon. de Syph.*, 1931, 6, 21.

[5] Bessemans, A., and De Moor, A., *Bull. Soc. Franc. de Dermat. et Syph.*, 1937, 4, 483.

[6] Van Haelst, J., C. R. Soc. de Biol., 1933, 113, 1535.

[7] Karrenberg, C. L., *Arch. f. Dermat. u. Syph.*, 1932, 165, 585.

TABLE I.

Results Obtained by Kato[3] in Darkfielding Nodes of Syphilitic Mice from One to 43 Days After Infection.

Days duration syphilitic infection	Treponemes in 250 darkfields per node	Days duration syphilitic infection	Treponemes in 250 darkfields per node
1	0	23	2
2	3	24	6
3	0	25	2
4	19	26	4
5	0	27	5
6	0	28	2
7	0	30	0
8	0	32	5
9	0	33	4
10	0	34	2
11	10	35	0
14	9	36	0
15	1	37	0
16	3	38	0
18	3	39	0
20	3	40	0
21	8	41	1
22	9	43	0

Total No. of mice, 36; total No. of Positive mice, 20.

would be only rarely necessary.

In this experiment, white mice were inoculated subcutaneously or intraperitoneally with Nichols rabbit strain of *Treponema pallidum*. Each mouse received 0.2 ml of rabbit chancre-emulsion containing more than 4 active treponemes per darkfield. A control rabbit inoculated with 0.5 ml of similar virus intratesticularly later developed a characteristic darkfield positive lesion.

The mice were kept in an air conditioned room at 65-70°F. They were housed in wire mesh cages with 4 to 10 mice per cage and were fed a proprietary food.† During the periods of observation, 45 and 90 days, unequivocal lesions of the mice were not observed. Forty-five and 90 days after infection, groups of mice were sacrificed and various organs from each mouse were emulsified in normal saline and inoculated into the testes of normal rabbits kept under similar conditions to the mice. The rabbits were examined at least twice weekly for the development of testicular lesions. Suspicious lesions were punctured and darkfielded. If characteristic motile treponemes were seen in the dark field, it was considered adequate proof that the mouse whose tissues had been subinoculated was infected with syphilis.

† Purina Laboratory Chow.

At the time these mice were sacrificed, iliac, axillary, or popliteal lymph nodes were dissected out and examined by Kato's method. The node was placed in a sterile petri dish and divided into 5 parts with a sharp scalpel. Each part was in turn placed in a drop of normal saline solution on a clean slide and emulsified by crushing and rotary movements of the flat surface of the scalpel blade. A cover slip was then placed on this emulsion, and 50 darkfields examined. A Spencer darkfield microscope with parabaloid condenser and built-in light was used. The objective was an oil immersion achromat with a numerical aperture usually of 0.8 being used together with a 10x ocular. Three observers examined the preparations. These all had considerable experience in recognition of the living *Treponema pallidum* by darkfield examination. All suspicious treponema-like objects were re-examined by the author.

The results as shown in Table II were completely negative. Because these results were obtained in an experiment mainly designed to study the biology of syphilis in the mouse, certain limitations existed which deserve discussion.

Actually, of the 36 mice employed, only 15 animals had darkfield examinations of a lymph node with simultaneous injections

TABLE II.
Comparison of the Results Obtained by Darkfielding Syphilitic Mouse Lymph Nodes with the Results Obtained by Inoculating Rabbit Testes with Mouse Tissues.

No. of mice	Route of infection	Duration of infection	Results of mouse organ* inoculation into rabbit testis	Results of mouse node inoculation into rabbit testis	Darkfield study of node†
5	Subcutaneous	45 days	4/5 Positive	—	Negative
11	,,	45 ,,	—	11/11 Positive	,,
4	Intraperitoneal	45 ,,	4/4 Positive	3/4 ,,	,,
6	,,	45 ,,	4/6 ,,	—	,,
10	,,	90 ,,	9/10 ,,	—	,,

* Either skin, blood, brain, spleen, or node, or a combination of these organs.
† Usually iliac node, rarely axillary or popliteal.

of other nodes into rabbit testes. Of these 15, none was infected intrascrotally, as Kato had done. Four of these 15 were infected by intraperitoneal injection, which is tantamount to intrascrotal injections in the mouse, which has free communication between the scrotum and the peritoneal cavity. It may seem from these statements that Kato's procedure was not duplicated. However, the important fact that 14 of the above 15 mice had chancrigenic nodes (for rabbits) suggests that the *Treponema pallidum* was present in the node that was simultaneously darkfielded. In addition, it is known from the work of many others[s] that once any syphilitic mouse organ is shown virulent for rabbit testis, it is extremely likely that lymph nodes of the same mouse also con-

tain the syphilitic virus. From this last statement, it follows that the 33 mice with positive rabbit subinoculation tests most likely had nodes containing the *Treponema pallidum*, and that Kato's procedure of performing darkfields was inadequate for demonstrating the organism. The existence of an invisible form of the syphilitic virus is not considered in this discussion.

Summary. 1. Attempts to use dark field examinations of the lymph nodes of mice as proofs of syphilitic infection 45 and 90 days after inoculation have failed, using the method employed successfully by Kato.

2. This failure to find the *Treponema pallidum* in lymph nodes by Kato's method confirms Karrenberg's studies.

3. More exhaustive methods of searching for the treponeme, such as those of Van Haelst, appear to be necessary.

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s Gueft, B., and Rosahn, P. D., *Am. J. Syph., Gon. and Ven. Dis.*, in press.

16106 P

Canine Epilepsy Caused by Flour Bleached with Nitrogen Trichloride (Agene). I. Experimental Method.[*]

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(Introduced by A. Earl Walker.)

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Many resports on canine hysteria have stressed the remarkable resemblance of this syndrome to human idiopathic epilepsy. The nervous disorder in dogs is characterized clinically by irregular "grand mal" seizures, "running fits," ataxia, and "hysterical" states, with apparent recovery between attacks. The electro-encephalograms of these dogs show a cerebral dysrhythmia similar to that of human epilepsy. Because of these common features, it seems proper to term the condition *canine epilepsy*.

A dietary etiology of this disease has been proposed by various investigators, *e.g.*, gliadin toxicity,[1] lysine deficiency from wheat flour diet[2] and wheat gluten flour toxicity.[3,4] This etiology is supported and explained by the crucial experiment of Mellanby.[5] He showed that the treatment of flour with agene gas, *i.e.*, nitrogen trichloride (NCl_3), a process used on approximately 90% of the white wheat flour milled in both England and the

United States, rendered that flour convulsant to dogs. The toxicity of the wheat fractions used in the investigations cited above was presumably due to agene treatment (NCl_3 treatment)[†] of the flour source, inasmuch as untreated wheat fractions showed no toxicity. The purposes of this communication are to describe a method of producing consistently and rapidly a convulsive disorder in dogs, and to delimit the toxic principle.

Method. A diet having a high flour component was prepared. The control ration, including supplementation with accessory food substances, and the actual analysis of this diet, is given below.

The diet, with water added, was baked into biscuits at 395°F for 30 minutes. The biscuits were canned and pasteurized after sealing. The analysis shown in Table I was made of these biscuits 2 weeks after baking.

The experimental ration was identical except that the flour component was heavily treated with agene. This was done in a bleaching unit in which the gas is generated by the interaction between chlorine gas and 1% ammonium chloride solution at a pH of 3.0. The NCl_3 gas thus formed was blown over the flour by means of an air stream, and was applied as a 1% solution of NCl_3 in air saturated with water vapor. Agene gas was used as the bleaching agent for all experimental flour samples, in a concentration of 30 g per 100 lb of flour, this value being determined by controlling the amount of chlorine gas available for reaction. No attempt was made to determine how much of the NCl_3 gas evolved had reacted with the flour.

1 Melnick, D., and Cowgill, G. R., *J. Nutrition*, 1937, 14, 401.

2 Arnold, A., and Elvehjem, C. A., *J. Am. Vet. Med. Assn.*, 1939, 95, 303.

3 Wagner, J. R., and Elvehjem, C. A., *J. Nutrition*, 1944, 28, 431.

4 Newell, G. W., Erickson, T. C., Gilson, W. E., and Elvehjem, C. A., PROC. SOC. EXP. BIOL. AND MED., 1947, 65, 115.

5 Mellanby, E., *Brit. Med. J.*, 1946, Dec. 14, No. 4484, 885.

TABLE I.
Basic Diet Used in Producing Canine Epilepsy.

Composition %		Analysis %
Casein	5	Protein	17.5
Liver powder	5	Carbohydrate	65
Salts IV	4	Fat	5
Sucrose	6	Ash	4
Corn oil	5	Moisture	8.5
Flour	75		
	100		100

Supplementation

	mg/kilo		mg/kilo
Thiamine	5.6	Thiamine	4.3
Riboflavin	5.6	Riboflavin	10
Pyridoxine	4		
Niacin	60	Niacin	94
Pantothenic acid	15		
Para amino benzoic acid	15		
Inositol	15		
Ascorbic acid	80	Ascorbic acid	120
Haliver oil	500		
α-tocopherol	100		

Results. When the control ration was fed to dogs of 5 to 10 kg weight, in quantities of 250 to 300 g per day, they gained weight, had a good appetite, and showed no symptoms of neurologic disorder or deficiency disease in the periods up to 45 days during which they were observed.

When the experimental diet (bleached flour) was fed to dogs, regardless of age, sex or the breed used, all the animals developed convulsions within 5 days, together with marked behavior disturbances and an ataxic gait. The electroencephalogram showed marked abnormalities, appearing between the 2nd and 3rd day of the experimental diet. No changes appeared after 45 days on the control diet. In all, 24 dogs were fed the experimental diet, and all 24 dogs developed canine epilepsy.

The wheat flour was fractionated after trials with numerous fat solvents showed that Bloor's mixture (75% anhydrous ethyl alcohol, 25% di-ethyl ether) was most satisfactory. By repeated extractions, it was possible to remove 1.4% fat from a straight grade of hard red winter wheat, the flour thus obtained containing less than 0.15% of fat by analysis. The solvent was removed from the fat at 100°C and a pressure of 20 mm Hg with a counter current of nitrogen, and both fat free flour and fat, were treated with agene. Feeding experiments showed that the toxicity remained unimpaired in the flour fraction, while the fat (although it became dark and rancid) did not prove toxic to test animals. Separation of gluten from the fat free flour showed that convulsive activity was retained in the protein fraction. Further confirmation of the relation between toxicity and the protein fraction is offered by the fact that casein treated with agene proved convulsant to 4 dogs.

The extraction of fat is emphasized, because commercially produced gluten has the lipid so firmly bound that it is not fully extractable at this stage. Hard red winter, hard red spring, and soft red spring wheat of clear, straight and patent grades all produced convulsions after treatment with agene although straight grade (so-called "family-grade") of hard red winter wheat was used in most of these experiments.

Monkeys were also used. The control diet seemed adequate to maintain nutrition in 6 monkeys for 3 weeks. The experimental diet did not produce convulsions, but the monkeys developed a tremor of the extremities and weakness of the hind limbs, within 5 days after changing to the experimental diet. This syndrome persisted 6 weeks later.

16107 P

Canine Epilepsy Caused by Flour Bleached with Nitrogen Trichloride (Agene). II. Role of Amino Acids.[*]

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Epilepsy in dogs fed bleached flour is attributable to the toxic effect of wheat proteins modified by nitrogen trichloride.[1,2] The heat stability of the toxic material, its presence in agenized gliadin and casein, and the rapid onset of symptoms[2] suggest that the toxicity resides in a relatively small molecule, possibly an altered amino acid. Thus it seemed advisable to test amino acids treated with nitrogen trichloride for convulsant properties.

The results of such tests in control and epileptic dogs are reported in the present work, changes in electroencephalographic pattern being used as a measure of convulsant activity. The electroencephalograms of the epileptic dogs prior to administration of amino acids also provide further insight into the syndrome.

Method. A mixture of synthetic amino acids was prepared to resemble the composition of gliadin. One-half of this amino acid mixture was kept as a control, and the other half was "bleached," *i.e.*, treated with agene (NCl$_3$) in the same manner as the flour.[2] The mixed amino acids were exposed for 10 minutes to 1% NCl$_3$ in air saturated with water vapor. No analysis for lost excess gas was performed. 3.3% solutions of the amino

acid mixtures were prepared by adjusting aqueous suspension to pH 10 with 10N NaOH, and then adjusting the pH to 8.5 with concentrated HCl. They were then used for intravenous injection.

Dogs were prepared under vinyl ether for artificial respiration, and for intravenous infusion *via* the femoral vein. The electrical activity of the brain and the electrocardiogram were recorded by means of Grass amplifiers and inkwriters. Paralysis was initiated by the intravenous injection of 25 mg dihydro-β-erythroidine hydrobromide and maintained by infusion of the erythroidine (1 mg per ml in 0.156 M sodium chloride). Under artificial respiration, the vinyl ether was rapidly eliminated and within 15 minutes the electroencephalogram resembled that of the intact animal. The dog was now available for testing the convulsant properties of the amino acid mixtures.

Results. Eight dogs on the experimental diet containing agenized flour (*i.e.*, bleached flour) for periods up to 2 weeks were used. These showed abnormal electroencephalograms but no seizures during a pre-injection period of 1 to 2 hours. The pattern illustrated in Fig. 1b is characteristic of all dogs on the experimental diet, and resembles that reported by others.[3]

Seizures followed the intravenous administration of the agene-treated amino acid mixture in less than 5 minutes (Fig. 1d, e, f). The absolute amount of amino acids varied

[*] The bleaching equipment was supplied through the kindness of the Wallace and Tiernan Company, Chicago. The synthetic amino acids and β-erythroidine were supplied through the kindness of Merck and Co., courtesy of Dr. J. M. Carlisle.

[1] Mellanby, E., *Brit. Med. J.*, 1946, Dec. 14, No. 4484, 885.

[2] Silver, M. L., Zevin, S. S., and Johnson, R. E., PROC. SOC. EXP. BIOL. AND MED., 1947, 66, 408.

[3] Newell, G. W., Erickson, T. C., Gilson, W. E., and Elvehjem, C. A., PROC. SOC. EXP. BIOL. AND MED., 1947, 65, 115.
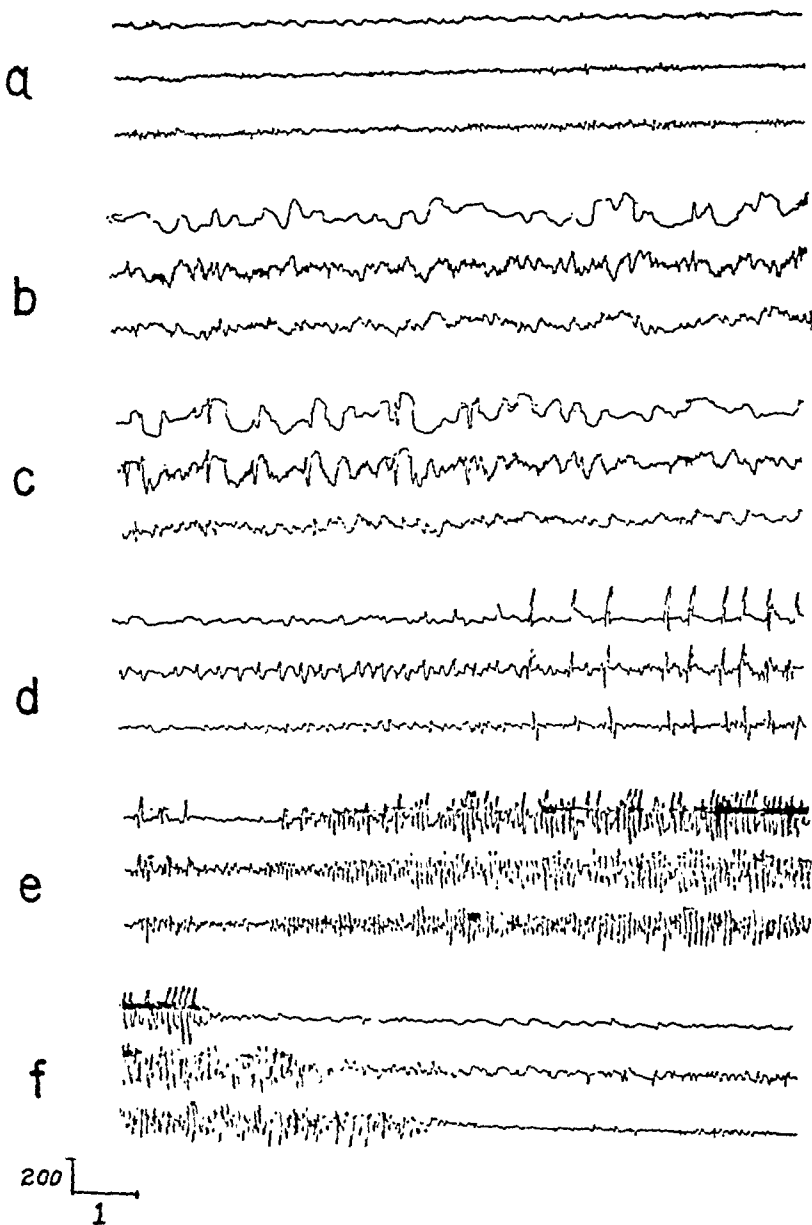
FIG. 1.

a. Normal electroencephalogram of dog on control (unbleached) diet. The upper lead is left parietal to ground; the middle lead is right parietal to ground; the lower lead is right parietal to left parietal (bipolar); this is constant in all tracings.

b, c. Abnormal E.E.G. of dog fed experimental (bleached) diet for three days. Note resemblance to petit mal activity.

d, e, f. In same dog as above, continuous record of a grand mal seizure occurring less than 5 minutes after the intravenous injection of "bleached" amino acid mixture. In small figure at lower left, voltage calibration is 200 μV, time calibration is 1 second. Each frame is ten seconds.

from 0.2 to 1.5 g. The injection of control untreated amino acids was ineffective in 4 dogs even after the injection of 20 g in 120 minutes.

In 12 dogs which were on the control diet containing untreated flour (*i.e.*, unbleached flour), the intravenous administration of the agene-treated amino acid mixture was followed by seizure patterns in the electroencephalogram of 4 dogs, marked abnormalities in 3 dogs, and no significant change in 5 dogs. In the animals with abnormal E.E.G.'s produced by intravenously administered agenized amino acids, the intracisternal injection of 1 to 2 cc of the NCl_3-treated amino acid mixture was followed by seizures within 5 minutes after injection.

Experiments have been conducted with individual amino acids treated in their crystalline states and the results have been negative

for arginine, glutamic acid, glycine, histidine, lysine, methionine, tryptophane and tyrosine; equivocal for proline, serine, and aspartic acid; and positive for cysteine hydrochloride. A possibility that should not be overlooked is that the toxic compound in foodstuffs' may be an altered polypeptide.

Summary. When an amino acid mixture approximating the composition of gliadin is treated with nitrogen trichloride gas (NCl_3) this mixture is so altered as to make it convulsant for dogs when given intravenously. The convulsant activity is most readily demonstrated in dogs which have been on a bleached (agenized) flour diet for 5 to 7 days, presumably because these animals have accumulated sufficient quantity of the toxic material *via* the alimentary tract, to make them highly susceptible to an additional increment received *via* the blood stream.

16108

Adrenal Cortical Activity in Urine of Horses.

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The presence of a substance in urine that protects adrenalectomized rats from the stress of cold was first noted by Anderson, Haymaker and Joseph[1] and Weil and Browne.[2] More extensive investigations have shown that extracts of urine from normal human subjects when tested in adrenalectomized rats

cause an elevation of liver glycogen,[3,4] increased life maintenance,[3,5] prolongation of muscle work performance,[6] and prevention of water intoxication.[7] Although the above studies have employed extracts of human urine, Selye and Dosne[8] have reported that the urine of large domestic animals contains cortin-like activity. The purpose of this in-

1 Anderson, E., Haymaker, W., and Joseph, M., *Endocrinology*, 1938, 23, 398.

2 Weil, P., and Browne, J. S. L., *Science*, 1939, 90, 445.

3 Venning, E. H., Hoffman, M. M., and Browne, J. S. L., *J. Biol. Chem.*, 1943, 148, 455.

4 Horwitt, B. N., and Dorfman, R. I., *Science*, 1943, 97, 337.

5 Dorfman, R. I., and Horwitt, B. N., *Fed. Proc.*, 1943, 2, 60.

6 Shipley, R. A., Dorfman, R. I., and Horwitt, B. N., *Am. J. Physiol.*, 1943, 139, 742.

7 Schiller, S., and Dorfman, R. I., *Endocrinology*, 1943, 33, 402.

8 Selye, H., and Dosne, C., *Lancet*, 1940, 239, 70.

TABLE I.
Liver Glycogen Response of Horse Urine Extracts.

Sample No.	Arg urine equiv. cc	Liver glycogen			Remarks
		Negative controls mg/100 g liver weight	Urine extract	Percentage increase %	
		Total extracts.			
B-18	200	37 (40)*	44 (22)*	+19	Normal urine
38-191	,,	33 (35)	38 (19)	+15	Pregnant mare
176-10	- ,,	45 (19)	53 (10)	+18	,, ,,
		Ketonic fraction			
38-174	400	40 (12)	80 (9)	+100	Normal urine
38-175-2	,,	80 (10)	90 (10)	+ 12	,, ,,
38-187	250	130 (15)	190 (10)	+ 46	,, ,,
38-191-1	330	30 (20)	100 (15)	+230	Pregnant mare
176-5-1	250	30 (10)	80 (10)	+170	,, ,,
		Non-ketonic fraction.			
38-174	200	60 (10)	60 (3)	0	Normal horse
38-175-2	,,	90 (15)	60 (9)	—33	,, ,,
38-187	,,	90 (15)	50 (9)	—45	,, ,,
38-191	,,	30 (15)	30 (9)	0	Pregnant mare

* Numbers in parentheses refer to number of mice employed.

vestigation was to assess the concentration of cortin-like activity in the urine of horses.

Method. The assay employed was a modification of the methods used by Venning, et al.[9] and Eggleston, et al.[10] Male white mice of the Swiss-Webster strain from our own stock colony weighing 20-25 g were used. At least a week before being used the mice were placed on a purified diet which contained 25% protein, 48% carbohydrate, 25% fat and 2% salt mixture,[11] plus adequate vitamin supplements. The animals were anesthetized with ether and adrenalectomized by the usual lumbar route. Following adrenalectomy they were placed in a warm room and given the above diet and 0.9% NaCl in place of their usual drinking water. On the morning of the fourth postoperative day food was removed and each mouse was given 25 mg glucose orally, and the first of 7 hourly injections of the extract under test. One hour after the last injection the mice were weighed and anesthetized with nembutal. The livers were quickly removed, weighed

and placed in hot 30% KOH for digestion. Glycogen was precipitated with alcohol and after hydrolysis, total reducing substances were determined by the method of Good, Kramer and Somogyi.[12] Total liver glycogen has been expressed as milligrams of glucose per 100 g of liver weight.

Urine Extracts. Urine was collected under toluene and acidified with acetic acid. All urines were extracted 3 times with one-quarter volume of ethylene dichloride or chloroform, usually employing a motor stirrer for the extraction. The combined extracts were then evaporated almost to dryness under reduced pressure and taken up in small amounts of 95% ethanol and diluted for injection or further fractionated by the method described by Venning. et al.[13]

Results. Table I lists the results obtained with 3 different types of extracts. Crude urine extracts that were not fractionated are referred to as total extracts. The ketonic and non-ketonic fractions were obtained by the Girard's reagent separation procedure.[13] Many of the total extracts of horse urine that have been prepared in this laboratory have shown evidence of toxic substances that resulted in a decrease in liver glycogen below the level of the negative controls. Dorfman,

9 Venning, E. H., Kazmin, V. E., and Bell, J. C., *Endocrinology*, 1946, 38, 79.

10 Eggleston, N. M., Johnston, B. J., and Dobriner, K., *Endocrinology*, 1946, 38, 197.

11 Bosshardt, D. K., Cierczsko, L. S., Buffington, A. C., and Arnow, L. E., *Arch. Biochem.*, 1945, 7, 1.

12 Good, C. A., Kramer, H., and Somogyi, M., *J. Biol. Chem.*, 1933, 100, 485.

13 Venning, E. H., Hoffman, M. M., and Browne, J. S. L., *Endocrinology*, 1944, 35, 49.

et al.[14] Venning, *et al.*[3] have commented on this toxic effect found in extracts of human urine. Fractionation evidently removes some of the toxic activity since glycogen responses are higher in the ketonic fraction than in the total extracts. As was to be expected no evidence of cortin-like activity was found in the non-ketonic fractions.

As a standard preparation for comparison, Wilson's aqueous adrenal cortical extract has been used. Comparing liver glycogen responses to Wilson's extract with the average response of the ketonic fraction of the horse urine extracts it was calculated that 1 liter of normal horse urine contains an equivalent of 0.10-0.15 cc of Wilson's extract and 1 liter of pregnant mare urine contains an equivalent of 0.30-0.40 cc of this extract. These figures compare favorably with results of Dorfman *et al.*[15] and Venning *et al.*[3] on human urine

extracts but appear to be definitely lower than the results reported for horse urine by Selye and Dosne.[8] The quantitative assay of the concentration of cortin-like material in urine is complicated by so many factors that the results presented here should be considered only as estimations.

Summary. The presence of cortin-like activity in extracts of horse urine has been confirmed. It is estimated that the concentration of this active material is approximately the same in urine of normal horses and normal humans. There is some indication that urinary cortin-like activity is increased in pregnancy.

14 Dorfman, R. I., Shipley, R. A., Schiller, S., and Horwitt, B. N., *Endocrinology*, 1946, **38**, 165.

15 Dorfman, R. I., Horwitt, B. N., and Fish, W. R., *Science*, 1942, **96**, 496.

16109

Nutrition of the Mouse. IV. Comparison of Bacterial Population of Two Highly Inbred Strains.*

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It was shown by Rogers, McElroy and Cowgill[1] that the nutritional requirements for reproduction and lactation differed for 2 strains of mice. Fenton and Cowgill[2] showed

1 Rogers, L. K., McElroy, L. W., and Cowgill, G. R., *Science*, 1942, **95**, 203.

2 Fenton, P. F., and Cowgill, G. R., *J. Nutrition*, 1947, **34**, 273.

the existence of a strain difference with respect to the riboflavin requirement for growth. Unpublished work in this laboratory has shown that this is also true of the pantothenic acid requirement. This dissimilarity of the nutritional requirements of 2 strains of mice may in part be due to different rates of intestinal synthesis of essential nutrients. The cecum has been thought to be an important site of the synthesis of B vitamins.[3,4,5,6] Man-

3 Guerrant, N. B., Dutcher, R. A., and Tomey, L. F., *J. Biol. Chem.*, 1935, **110**, 233.

4 Taylor, A., Pennington, D., and Thacker, J., *U. of Texas Pub.*, 1942, **4237**, 135.

5 Schweigert, B. S., McIntire, J. M., Henderson, L. M., and Elvehjem, C. A., *Arch. Biochem.*, 1945, **6**, 403.

6 Mannering, G. J., Orsini, D., and Elvehjem, C. A., *J. Nutrition*, 1944, **28**, 141.

TABLE I.
The Weight of the Cecal Contents and the Bacterial Count per Gram of Cecal Contents and per Cecum of the 2 Strains of Mice.

Strain	Diet	No. of animals	Wt of cecal contents, g	Bacterial count	
				per cecum, billions	per g of cecal contents, billions
A strain	101	12	.065 ± .021*	1.5 ± .84	22.2 ± 11.5
C$_{57}$ strain	101	12	.176 ± .047	5.11 ± 2.62	28.7 ± 11.5
Young A strain	stock	3	.386 ± .038	13.12 ± 3.03	32.4 ± 4.34
Young C$_{57}$ strain	,,	3	.352 ± .070	8.39 ± 1.76	23.9 ± 2.1
Older A strain	,,	6	.417 ± .045	19.86 ± 6.1	48.0 ± 16.8
Older C$_{57}$ strain	,,	3	.406 ± .125	15.1 ± 5.9	37.0 ± 13.4

* Standard deviation.

nering et al.[6] worked with rats fed synthetic diets containing various carbohydrates and state that with certain exceptions the riboflavin found in the feces was related to the dry weight of the cecal contents.

The purpose of this investigation was to study the intestinal flora of 2 strains of mice, one with characteristically high susceptibility to spontaneous mammary tumors (strain A) and the other with low susceptibility in this respect (strain C$_{57}$). These two strains were chosen because they had been used in previous studies in this laboratory.

Experimental. The 39 mice used in this study had been maintained on one of 2 diets: a synthetic ration containing dextrose as the carbohydrate without added biotin or folic acid (diet 101 as described previously[7]) or a stock ration.† Twelve mice of the C$_{57}$ strain (tumor-resistant) and 12 of the A strain (tumor-susceptible) ranging in age from 2 to 4 months were used after having been maintained on the synthetic ration. The remainder of the animals had been fed the stock diet and fell into 2 age groups, namely, 2-2.5 months and about 12 months old. The mice were sacrificed by etherization and the cecal contents removed and weighed. Bacterial counts and cultural work were carried out as described in another publication.[8]

Results. As shown in Table I, the mice of the A strain on the synthetic diet had smaller

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cecal contents than the C$_{57}$ mice on the same diet. In only one case did the highest value given by an A strain mouse equal the lowest weight obtained from a C$_{57}$ animal. The differences in weights of cecal contents between these 2 groups was found to be statistically highly significant (p < 0.01). The total bacterial count per g of cecal contents was about the same in both strains. However, the effect of the larger cecal contents of the black mice was reflected in the greater total bacterial population of the cecum of this strain when compared to the white animals. This difference was also found to be statistically highly significant (p < 0.01).

The results obtained by studying the Gram stains and the cultures isolated from the ceca of both strains were similar with respect to the occurrence of cocci and Gram-positive rods. However, there appeared in the highest dilutions of the C$_{57}$ mice in 10 cases out of 12 a short Gram-negative, anaerobic rod. This type of organism occurred in only 2 white mice out of 12. Coliform organisms were encountered slightly more frequently in high dilutions of cecal contents of the A strain mice (5 out of 12 animals) than in the C$_{57}$ strain (2 out of 12 mice).

These two strains of mice, when fed a stock ration, showed no differences in weights of cecal contents and total bacterial population per cecum. The lower values shown by C$_{57}$ mice for bacterial counts per g of cecal contents and per cecum were not considered significant owing to the wide variation in counts and to the small number of animals in each series. On microscopic examination of the

7 Fenton, P. F., and Cowgill, G. R., J. Nutrition, 1947, 33, 703.

† Purina Laboratory Chow.

8 Gall, L. S., Fenton, P. F., and Cowgill, G. R., J. Nutrition, in press.

Gram stain and from cultural results the bacterial flora of the cecum of the older animals of both strains seemed to be similar, but there was a tendency for the younger black animals (C_{57}) to have a more varied flora than was shown by the white mice of the same age. It is unsafe to draw a general conclusion from so small a series of mice, even though this difference was observed on all animals.

In Table I the values for the stock ration animals are broken down into groups of young and older mice, so that these stock animals may be compared with those on diet 101. As was pointed out in a previous paper by these authors,[8] the weight of the cecal contents and the bacterial count per g and per cecum were considerably higher in the A strain mice on the stock diet than in the A strain mice fed a synthetic ration. By inspection of the data in Table I, it is evident that even though the black animals fed a synthetic diet had greater cecal contents and higher bacterial counts per cecum than the A strain mice, they still fell short in these respects of the C_{57} animals of the same age on the stock diet.

Discussion. There has been no really satisfactory explanation offered for the strain differences in requirement for certain of the B vitamins, as manifested by mice of the C_{57} and A strains. (Grüneberg[9] discussed the various anatomical and physiological differences in the alimentary tracts of mice of several strains, but there is no mention made of any variations in the amount of cecal contents, or in the size of the cecum. It is therefore of interest to note that in our study a significant difference in the weight of the cecal contents of 2 strains of mice was found when these animals were fed a synthetic diet, especially since the A strain mice, which had the smaller cecal contents also had a higher dietary requirement for certain B vitamins.

It has been shown[3,4,5] that the cecum is one of the sites of bacterial synthesis of B vitamins in the intestinal tracts of rats. For this reason it may well be that the larger bacterial population offers an explanation for the lower requirements of the C_{57} strain for certain B vitamins. Further support for this theory is found in the cultural work done on the cecal contents of animals fed this synthetic diet in which it was demonstrated that bacteria isolated from top dilutions of these contents in numbers of 100 million or more per gram were able to synthesize some of the B vitamins *in vitro*.[10]

Summary and Conclusions. 1. Studies were made to determine whether there is a difference in the weight and bacterial population of the cecal contents of mice of the C_{57} and A strains, which were the same age and received the same diet.

2. The C_{57} animals fed a synthetic diet (No. 101) had a significantly greater amount of cecal contents and bacterial count per cecum than the A strain mice of the same age and fed the same diet. Although most of the bacterial flora appeared to be the same in the 2 strains, a Gram-negative, anaerobic rod did occur more frequently in the C_{57} mice than in the A strain.

3. There was no significant difference between the black and white mice of the same age fed the stock ration with respect to weight of cecal contents or bacterial count per cecum. The young black mice may have had a more diverse flora than the white mice of the same age.

4. It was postulated that this greater weight of cecal contents with its higher bacterial population in the C_{57} mice might help to explain the lower B vitamin requirements of this strain when comparing it with animals of the A strain, both fed the same synthetic diet.

9 Grüneberg, Hans, *Genetics of the Mouse*, 1943, Cambridge University Press, London, England.

10 Gall, L. S., Illingworth, B. A., Cowgill, G. R., and Fenton, P. F., *J. Nutrition*, in press.

16110

Spasmolytic Action of Dolophine (Amidone).[*]

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(Introduced by Nilkanth M. Phatak.)

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Amidone (6 dimethylamino-4, 4, diphenyl-3-heptanone) is a new synthetic analgesic compound related to demerol (Dolantin) originally prepared at the I.G. Farben-industrie research plant at Hochst am Main and first described in this country in the U. S. State Department Technical Intelligence Team Report.[1] Scott and Chen[2] reported that this agent had marked analgesic properties when tested in both dogs and humans by the Wolff-Hardy technic as well as a comparatively low toxic/analgesic ratio. Later, these workers named the compound "dolophine" and reported the clinical trial of 4 related compounds.[3] They also found that "dolophine" possessed about equal spasmolytic action when compared with demerol on isolated intestinal strips. Karr[4] found decreased propulsive activity of the intestines of rats and rabbits after "dolophine" which was slightly more marked than the decrease noted after demerol. We have further studied the action of these spasmolytic agents on isolated intestinal strips and, in addition, have compared their effects on the isolated uterine musculature of rabbits and rats.

Method. The usual technic for recording contractions of isolated muscle strips was used. Furfuryl trimethyl ammonium iodide (Furmethide)[5] served as the spasmogenic agent for the rabbit intestinal strips and the rat uteri. Methyl ergonovine (Methergine)[6] was used to stimulate contraction in rabbit uterine strips. Other agents were also used for stimulating contraction.

Results. "Dolophine" and demerol produced about the same degree of spasmolysis on the isolated rabbit intestine when equal concentrations of the drugs were used, with concentrations as low as 0.025 mg/50 cc (1:2,000,000) being effective. These results for demerol agree with other reports.[7,8,9]

The contracted rat uterus showed a similar relaxation when concentrations of either drug as low as 1:2,000,000 were used. Rabbit uteri, however, required more "dolophine" than demerol for spasmolysis on most trials, the least effective concentration being 0.075 mg/50 cc (1:666,666). Normally active smooth muscle strips were found to respond irregularly to both drugs.

The pharmacodynamic basis for the spasmolytic action of these compounds is not fully known. Since "dolophine" lysed spasm induced by both musculotropic (Methergine, Barium, Pitressin) and neurotropic (Furmethide, Neostigmine, Acetylcholine) spasmogenic agents, its action is probably largely musculotropic. In this respect it resembles trasentin, which, in addition to a musculotropic spasmolytic action, shows some local anesthetic properties. A recent report[10] in-

1 Kleiderer, E. C., Rice, J. B., Conquest, V., and Williams, J. H., Report 981, Office of the Publication Board, Department of Commerce, Washington, D.C.

2 Scott, C. C., and Chen, K. K., J. P. E. T., 1946, 87, 63.

3 Scott, C. C., Kohlstaedt, K. G., and Chen, K. K., Anesth. and Analges., 1947, 26, 12.

4 Karr, N. W., Fed. Proc., 1947, 6, 343.

5 Fellows, E. J., and Livingston, E. A., J. P. E. T., 1940, 58, 231.

6 Kirchhof, A. C., Racely, C. A., Wilson, W. M., and David, N. A., West. J. Surg., Gyn. and Obs., 1944, 52, 197.

7 Yonkman, F. F., Noth, P. H., and Hecht, H. H., Ann. Int. Med., 1944, 21, 7.

8 Climenko, D. R., Fed. Proc., 1942, 1, 15.

9 Yonkman, F. F., Anesth. and Analges., 1944, 23, 207.

10 Way, E. L., Science, 1945, 101, 566.

dicates that demerol, like trasentin, has local anesthetic properties and Everett[11] in this laboratory has demonstrated that "dolophine" produces local anesthesia in the rabbit's cornea. Thus, the local anesthetic property, as well as musculotropic spasmolysis seems to be common to trasentin, demerol and "dolophine."

Summary. Isolated strips of rabbit intestine and rat and rabbit uteri respond with spasmolysis when treated with "dolophine" and demerol when spasm is induced with furmethide or Methergine. The ratio of spasmolytic concentration is approximately 1:1 although rabbit uteri required slightly more dolophine than demerol for spasmolysis.

11 Everett, F. G., personal communication.

16111 P

Induction of Male Copulatory Behavior in the Brown Leghorn Hen.*

L. V. DOMM AND BEN B. BLIVAISS.

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Previous experiments have shown that daily injections of testosterone propionate in poulards[1] and pullets[2] will induce masculinization of somatic sex characters and behavior. These individuals crowed, became combative, and displayed male courtship but no copulatory behavior, even though injections were continued for periods up to 7 months. The absence of this phase of behavior has long baffled investigators in view of the ease with which other sexual characters may be masculinized in the fowl. Two incidental cases are recorded[3,4] in which hens revealed male copulatory behavior, although neither instance is based on extensive experimentation. The present experiments, in progress for over 4 years, show that masculine copulatory behavior may be induced and maintained in some pullets following successive implantations of androgen pellets.

Each of 4 non-laying pullets (A, BW, G, and GA) received subcutaneous implantations of testosterone propionate pellets (average weight, 33.4 mg) when 5 months old and subsequently at 84, 164, 244, 337, 425, 530, 630, and 727 days. Two control pullets (B and W) received implantations of cholesterol pellets averaging 32.6 mg. Pellet implantations were repeated upon absorption, determined by palpation and comb regression. The behavior was tested, generally on alternate days, by introducing each bird, alone in its pen, to a female, to a male, or to a dummy female mounted in the squatting, copulatory position.

Experimental pullet A was first observed to crow at 34 days, to chase, grab, and attempt mounting at 95 days, to grab, mount, and copulate at 110 (age 262), and to waltz and circle at 114 days after the first implant. Pullet BW was also first observed to crow at 34, chase, grab, and attempt mounting at 95, mount and copulate at 103 (age 255), and waltz and circle at 127 days. A continued to copulate for 5 months, when it died, and BW for 8 months after the final implant. Courtship activity preliminary to copulation, never very vigorous, was frequently absent. In no instance did they indicate receptivity by squatting at the approach of the male.

1 Davis, David E., and Domm, L. V., PROC. SOC. EXP. BIOL. AND MED., 1941, 48, 667; *Essays in Biology*, University of Calif. Press., 1943, 171.

2 Domm, L. V., Davis, David E., and Blivaiss, Ben B., *Anat. Rec.*, 1942, 84 (Suppl.), 31.

3 Domm, L. V., *J. Exp. Zool.*, 1927, 48, 31.

4 Zitrin, A., *Endocrinology*, 1942, 31, 690.

The other pullets (G and GA) displayed courtship ·but' no masculine copulatory behavior during the period of observations.

In the second group, 6 pullets received their first pellet at one month and further implants (average, 25.9 mg) at 109, 202, 297, 395, 495, and 592 days thereafter. One bird, G, was first observed to crow at 125, chase at 126, waltz and circle at 141, and to mount and copulate at 161 days (age 191) after the first implant. Sporadic copulations occurred until 11 months after final implantation. The other 5 birds began to crow and display courtship at approximately the same time as G but did not mount or copulate.

In a third group, 8 pullets received pellet implantations beginning at 4 days and additional implants (average, 23.2 mg) at 62 and 172 days. In this group, only one bird crowed and another was observed to waltz and circle.

In the fourth group, 6 pullets received the first pellet when 3 months old and further implants (average, 28.2 mg) at 80, 185, 317,† and 436 days subsequently. All were known to crow and 3 displayed courtship behavior. No copulations were observed.

A standard trap-nest was kept in all pens throughout the experiments. One treading pul-

† At the time of the second, third, and fourth implantations, 3 of the pullets received 2 pellets each. The average weight of both was 57.1 mg.

let (A) never laid. It died 871 days after first implantation. The other treader (BW) laid 6 eggs between the 69th and 80th days following second implantation, and 3 more after a lapse of one year following the last implant. During the 4 years of observation the non-treading pullet G laid 6 eggs between 57th and 74th days, and GA only one egg on 79th day after second implantation.

In the second experiment, treading hen G, as well as 3 non-treaders, never laid over a span of 3 years. One non-treading hen laid an egg at 4 months after the first, and another laid one egg at 2 years after the last implant. Similar records of sporadic egg-laying were obtained in the other experiments, while some controls laid as many as 21 eggs per month.

Summary. Three cases are described from 4 experimental groups totaling 24 birds, some observed for more than 4 years, in which single comb brown Leghorn hens displayed male copulatory behavior following successive implants of testosterone propionate pellets. Implantations were begun when birds were 4 days, and 1, 3, and 5 months old. These results contrast with earlier negative findings following single daily injections of this hormone. Induction of copulatory behavior in such a small percentage of cases indicates the desirability for further study of factors controlling this behavior pattern.

16112

Incidence of Rh Factor Among the Indians of the Southwest.

Earl B. Gerheim.* (Introduced by John H. Ferguson.)
From the Department of Physiology, University of North Carolina School of Medicine, Chapel Hill, N. C.

Within the last 12 years the Indians of the Southwest have been investigated as to the occurrence of the various agglutinogens. Allen and Schaeffer[1] reported on the classic blood groups, and Allen and Larsen[2] reported their

* Junior Research Fellow from the National Institute of Health.

findings on the M and N agglutinogens. The basis of this report is the Rh factor among the above mentioned Indians.

[1] Allen, F. W., and Schaeffer, W., Univ. N. Mex. Bull. Biol. Series. 1935, 4, 2.

[2] Allen, F. W., and Larsen, H. D., J. Immunol., 1937, 32, 301.

The Indians tested were patients at the U. S. Indian Hospital and the Indian School Hospital at Albuquerque, New Mexico. Students of the Indian School at Albuquerque and the Day School at Isleta were also used in this study. The age range was from school age to 60 years.

Lederle's Anti-Rh Serum (Anti-Rh$_o$) was used in this series of tests; this serum is of animal origin. Two drops of blood to be tested were added to 0.5 cc of physiological saline containing 1% sodium citrate. One drop of the cell suspension is placed on a clean glass slide. Next to this is placed one drop of Anti-Rh Serum. The two are mixed with a glass rod and finally by tilting the slide back and forth once. The slide is placed undisturbed on the table for 3 minutes. At the end of this time the slide is picked up and given 2 or 3 gentle turns. While continuing to hold the slide a gentle motion is maintained and the test mixture is examined macroscopically with oblique lighting until the end of 6 minutes. Clumping is always visible within 6 minutes in positive tests and all observations must be terminated in 6 minutes. It is necessary to guard against rapid evaporation in dry air; a Petri dish was used to cover the slides. Rh-positive and Rh-negative controls were run with all tests.

The census records of the United Pueblo Agency at Albuquerque were used to determine the racial purity of the Indians concerned. Table I records the findings of the full-blooded (4/4) Indians tested.

Seven Rh positives were found among Indians other than full bloods (4/4). The range of racial purity ran from 2/4 to 15/16.

TABLE I.
Incidence of Rh Factor Among Full-Blooded (4/4) Indians Tested.

Tribe or Pueblo	Rh positive	Rh negative
Acoma	62	0
Apache	8	0
Cochiti	6	0
Hopi	6	0
Isleta	75	0
Jemez	9	0
Laguna	70	0
Navajo	53	1
Sandia	6	0
San Felipe	22	0
San Ildefonso	1	0
San Juan	6	0
Santa Ana	10	0
Santa Clara	3	0
Santo Domingo	19	0
Sia (Zia)	8	0
Taos	12	0
Tesuque	3	0
Tewa	1	0
Zuni	5	0
Totals	385	1

In some cases census records were unavailable; there were 55 Indians in this category. All were Rh positive.

The total findings are 447 Rh positives and one Rh negative in the Indians tested.

Similar results have been obtained by other investigators[3,4] in testing Indians of North America.

3 Landsteiner, K., Wiener, A. S., and Matson, G. A., J. Exp. Med., 1942, 76, 73.

4 Wiener, A. S., Zepeda, J. P., Sonn, E. B., and Polivka, H. R., J. Exp. Med., 1945, 81, 559.

16113

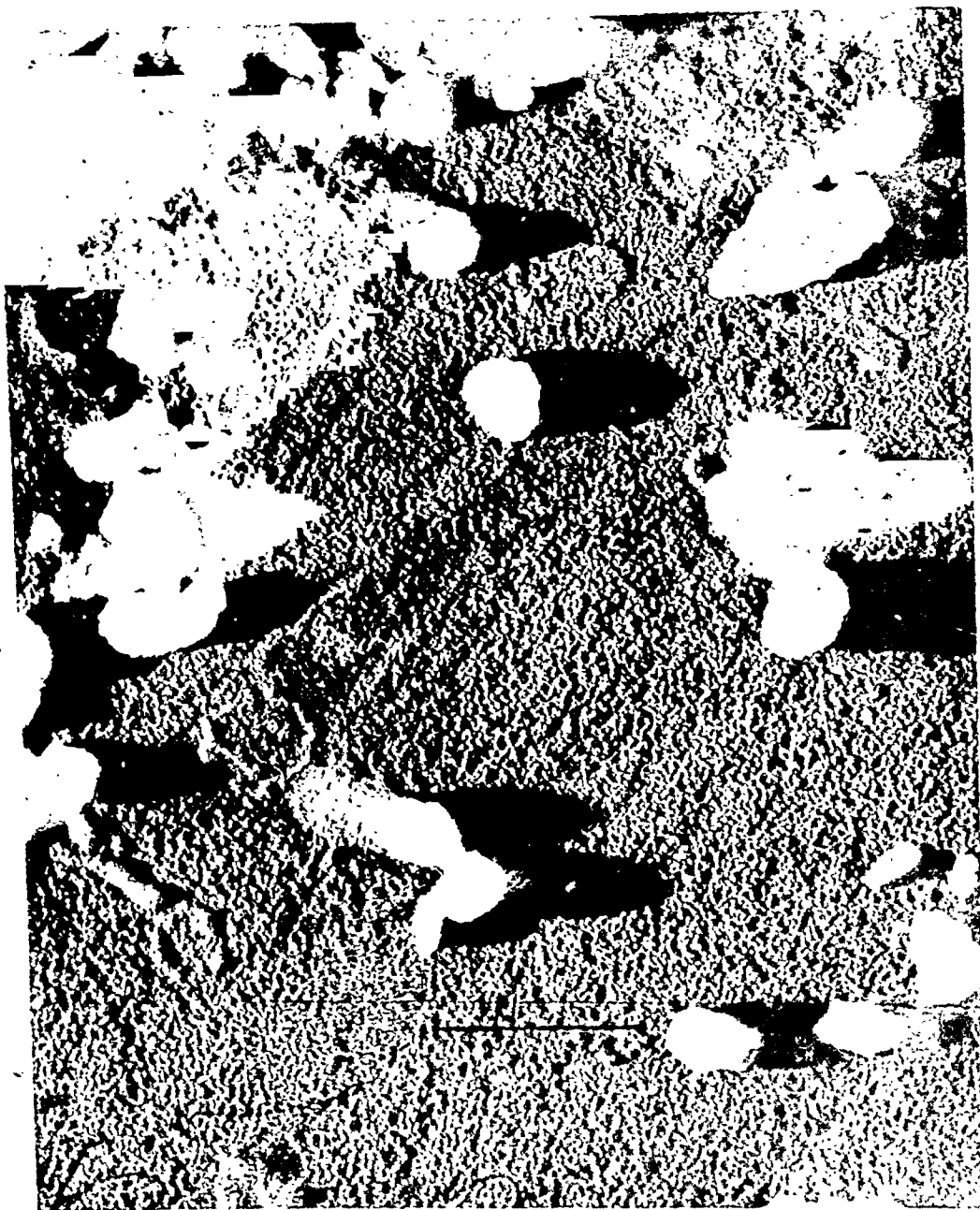# Chemistry of Melanin. IV. Electron Micrography of Natural Melanins.[*]

Howard S. Mason, Herbert Kahler, Ross C. MacCardle, and Albert J. Dalton.

From the Office of Dermatology, Industrial Hygiene Division, and the National Cancer Institute, National Institute of Health, U. S. Public Health Service, Bethesda, Md.

Although natural melanins apparently consist of insoluble, heterogeneous aggregates[1,2,3] a water-soluble melanin-containing pseudoglobulin has been separated from pigmented tissue.[4] In this study, we have examined both melanin granules and the melano-pseudoglobulin with the electron microscope to determine (a) whether melanin consists of formed elements or amorphous aggregates, (b) whether structure upon the surface of and within the granule is discernible, and (c) what formal relationship exists between melano-pseudoglobulin and the natural pigment.

Experimental and Results. Electron micrographs were taken at a magnification of 7,600 diameters with a Universal model (R.C.A.) electron microscope. Collateral optical microscopy was carried out with a Zeiss phase contrast microscope† at a magnification of 1085 diameters. Samples of 50 granules were measured to determine size ranges.

Melanin granules from the S-91 transplantable mouse melanoma. Tissue‡ (4-5 g) was ground for 15 minutes with an equal volume of sand and of chilled isotonic saline buffered with 1% of Sorensen's phosphates to pH 6.9. The mixture was diluted to 150 ml with cold saline, then centrifuged at 5° as shown in Table I.

Under the phase contrast microscope the

TABLE I.

Time (minutes)	Force (X gravity)	Sediment	Supernatant
3	74	Fraction 1	recentrifuged
15	74	,, 2	,,
15	159	,, 3	,,
15	283	,, 4	,,
15	423	,, 5	,,
15	636	,, 6	,,
15	1130	,, 7	,,
30	1130	,, 8	Fraction 9

low speed fractions 2-4 appeared to contain large aggregates and amorphous clumps of both pigmented and non-pigmented materials. In fraction 5 a large majority of the particles were pigmented and in the one μ size range; a small number of aggregates of these granules were also present. In addition, some particles were found which were almost invisible in ordinary light but were demonstrable by phase contrast. The proportion of dark pigmented granules to these "grey" particles was approximately 80/20. Other, highly refractile granules variable in size and shape and ranging from 2 μ to 4 μ were occasionally seen. In fractions 6-9, unit granules comprised the major portion of the particulate material but the proportion of melanin granules to "grey" particles gradually decreased.

Fraction 5 was resuspended in 5 ml of cold distilled water and centrifuged for 15 minutes at 74x gravity. The supernatant fluid was then centrifuged for 15 minutes at 423x gravity. The sediment so obtained, resuspended in cold distilled water, proved to be the most homogeneous preparation of melanin granules obtained from the S-91 melanoma. Roughly 80% of all the elements present were shown by phase contrast to be pigmented. Under the electron microscope this fraction was found to consist principally of elliptical particles, the major axes of which ranged from 0.30 μ to 0.45 μ and the minor axes from

* For the last paper in this series, see Mason, H. S., *J. Biol. Chem.*, Jan., 1948, in press.

1 Smith, D. T., *Bull. Johns Hopkins Hosp.*, 1925, 36, 185.

2 Herrmann and Boss. M. B., *J. Cell. and Comp. Physiol.*, 1945, 26, 131.

3 Mason, H. S., *Ann. N. Y. Acad. Sci.*, in press.

4 Greenstein, J. P., Turner, F. C., and Jenrette, W. V., *J. Nat. Cancer Inst.*, 1940, 1, 377.

† Loaned by the Army Air Forces, Wright Field.

‡ The tumor material was contributed by Dr. Glenn H. Algire.

- The Indians tested were patients at the U. S. Indian Hospital and the Indian School Hospital at Albuquerque, New Mexico. Students of the Indian School at Albuquerque and the Day School at Isleta were also used in this study. The age range was from school age to 60 years.

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San Felipe	22	0
San Ildefonso	1	0
San Juan	6	0
Santa Ana	10	0
Santa Clara	3	0
Santo Domingo	19	0
Sia (Zia)	8	0
Taos	12	0
Tesuque	3	0
Tewa	1	0
Zuni	5	0
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3 Landsteiner, K., Wiener, A. S., and Matson, G. A., J. Exp. Med., 1942, 76, 73.

4 Wiener, A. S., Zepeda, J. P., Sonn, E. B., and Polivka, H. R., J. Exp. Med., 1945, 81, 559.

Fig. 2.
Melanin granules from the S-91 melanoma, gold-shadowed at an angle of 1 to 5.

The fractions obtained corresponded in content to those from the S-91 melanoma, and under the electron microscope these melanin granules appeared identical in size range and shape to those from the S-91 melanoma: no internal structure was revealed. (Fig. 3, 4).
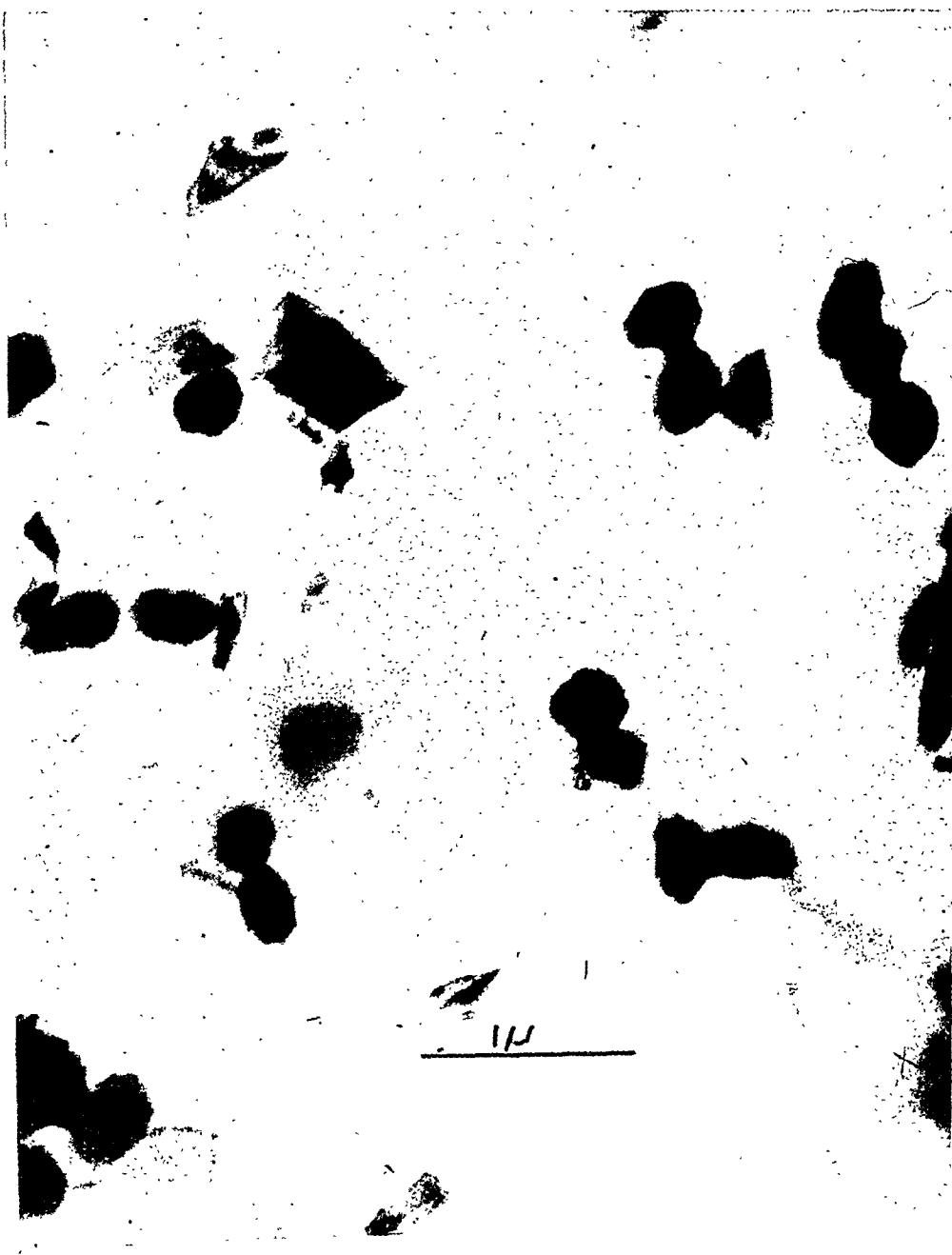
FIG. 1.
Melanin granules from the S-91 melanoma.

0.25 μ to 0.40 μ. The melanin granules themselves appeared almost opaque to the electron beam and no internal structure could be discerned (Fig. 1, 2).

Melanin granules from the Harding-Passey transplantable mouse melanoma. The process utilized above proved effective in concentrating the pigment granules from this source.[*]

FIG. 4.
Melanin granules from the Harding-Passey melanoma, gold-shadowed at an angle of 1 to 5.

tion but clumped material and unit particles ranging from 0.5 μ to 7 μ were observed. Under the electron microscope, few of these particles corresponded in size or shape to melanin granules from the parent melanoma. *Melanin granules from beef iris, ciliary body*
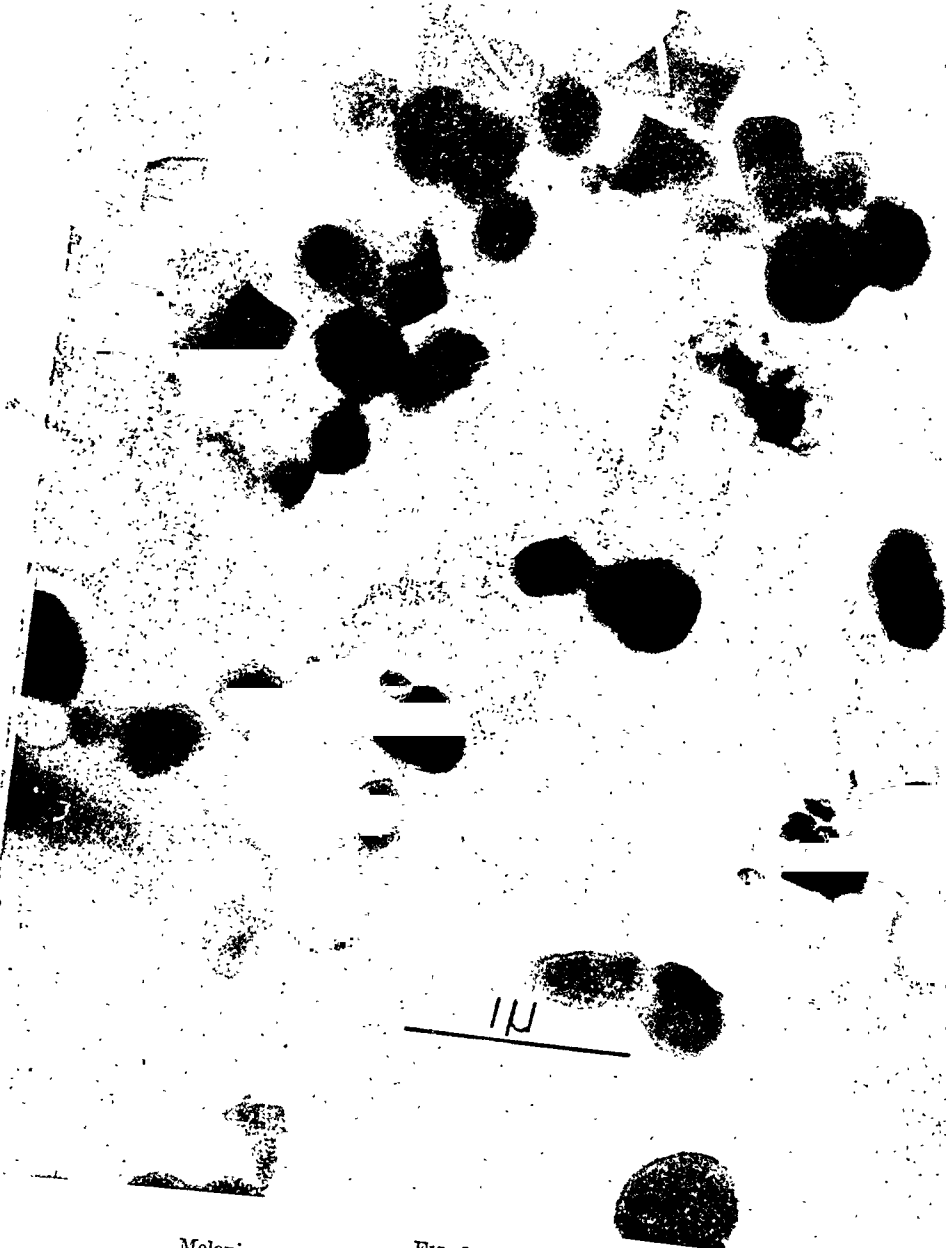
FIG. 3.

Melanin granules from the Harding-Passey melanoma.

Amelanotic melanoma. The specimens[5]‡ were prepared and centrifuged in the manner described for the two tissues above. In the phase contrast microscope no evidence of pigmented granules could be found in any frac-

[5] Algire, G. H., *J. Nat. Cancer Inst.*, 1944, 151.
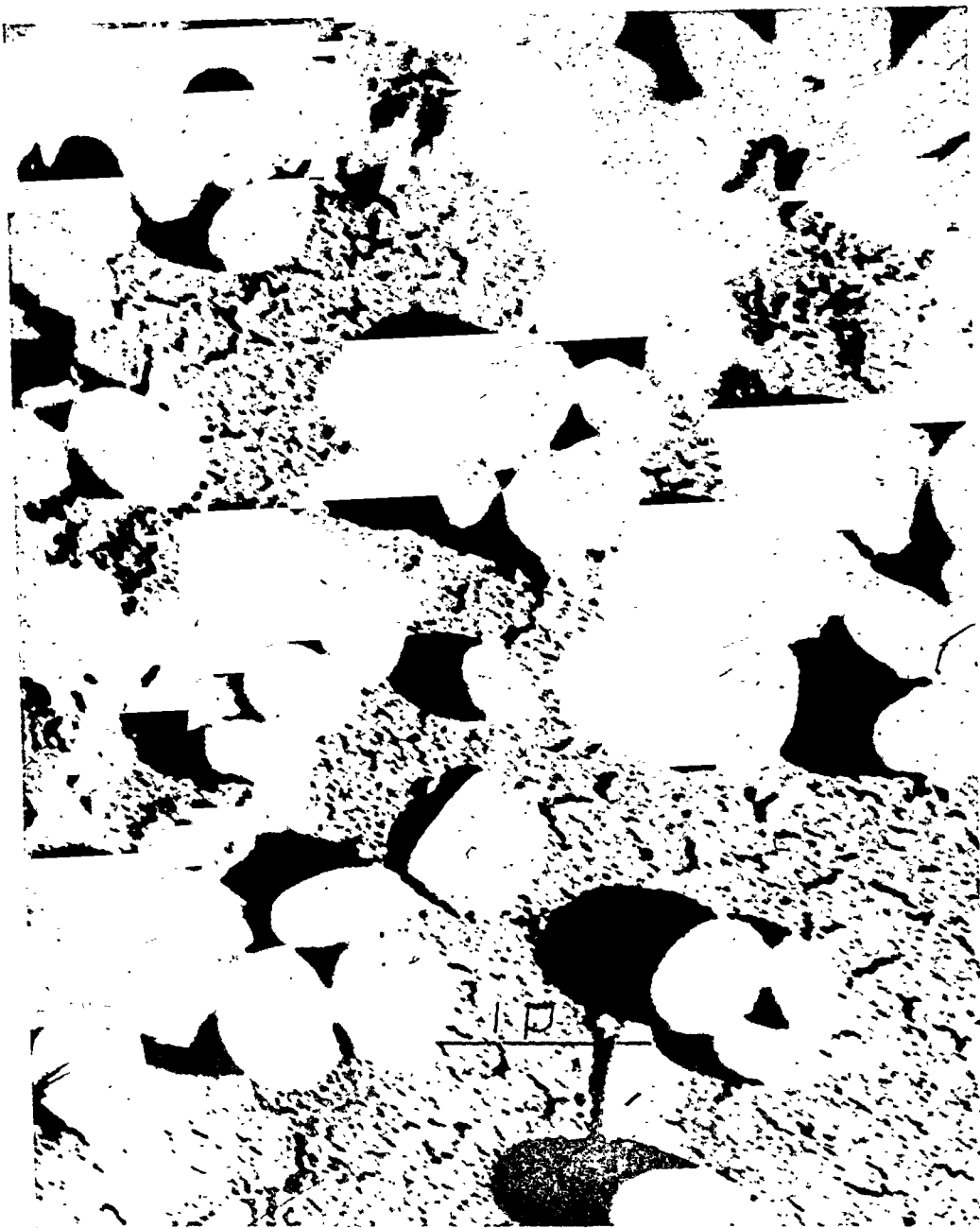
FIG. 6.
Melanin granules from the beef choroid, gold-shadowed at an angle of 1 to 5.

saline as before and the suspensions centrifuged at 74x gravity for 15 minutes; the pellets were resuspended in cold saline and recentrifuged at the same speed. The combined supernatant fluids were spun at 3672x gravity for 30 minutes, and the pellets in each case were resuspended in 10 ml of cold distilled water. The ciliary body preparation then

FIG. 5.
Melanin granules from the beef choroid.

and choroid. Ciliary body, iris and choroid were separated from twenty beef eyes.[§] Each

tissue was ground with sand and cold buffered

6 Friedenwald, J. S., Herrmann, H., and Moses, R., *Bull. Johns Hopkins Hosp.*, 1943, **73**, 421.

§ Beef eyes were obtained from freshly slaughtered animals through the courtesy of the Schluderberg-Kurdle Corp.

FIG. 8.
Melanin granules from human skin, gold-shadowed at an angle of 1 to 5.

The particles in each preparation were further sorted by removing aggregated material at 74x gravity (15 minutes), then depositing the major portion of the melanin at 423x gravity (15 minutes) and resuspending the pellet in 5 ml of distilled water. Under the

FIG. 7.
Melanin granules from human skin.

corresponded to the granule preparation of Herrmann and Boss.[2] Under the phase contrast microscope it was observed to consist of approximately 95% melanin granules which varied widely in shape and size, together with extraneous material including colorless nuclei.

FIG. 8.
Melanin granules from human skin, gold-shadowed at an angle of 1 to 5.

The particles in each preparation were further sorted by removing aggregated material at 74x gravity (15 minutes), then depositing the major portion of the melanin at 423x gravity (15 minutes) and resuspending the pellet in 5 ml of distilled water. Under the

phase microscope the preparations appeared homogeneous with respect to melanin granules. In the electron microscope the granules varied from circular to elliptical shape, 0.30 μ to 2.00 μ across; they were sharply defined but again failed to reveal internal structure (Fig. 5, 6).

Melanin granules from human skin. A fresh specimen of colored human skin measuring 6 cm by 22 cm was obtained through the courtesy of Drs. John Wirth and Emerson Y. Gledhill. The heavily pigmented epidermis was readily scraped away from the dermis with a blunt scalpel and ground with 2 g of white sand and 5 ml of cold buffered saline for 15 minutes. The resultant slurry was diluted with 25 ml of chilled saline and centrifuged for 15 minutes at 74x gravity. The supernatant fluid was then centrifuged for 15 minutes at 423x gravity. All pigment was deposited in the pellet, which was resuspended in cold distilled water and cleared of aggregated material by centrifuging at 74x gravity for 15 minutes. Under the phase contrast microscope colorless globules about one μ in diameter were observed to constitute about 10% of the bodies visible. Of the pigmented particulates about 35% were rod-shaped and another 35% were small spheres of uniform shape and size. The remainder of the particulate material appeared as large clumps of dark granules. Under the electron microscope globular, rod-shaped, and clumped elements were observable (Fig. 7, 8). The rods varied in size from 0.10 μ x 0.40 μ to 0.18 μ x 0.60 μ. The pigmented globules appeared to have diameters between 0.20 μ and 0.30 μ. The photographic images were uniformly dense and no indication of internal structure could be discerned.

The melanin-containing pseudoglobulin from the S-91 melanoma. This material was prepared by following the directions of Greenstein, Turner, and Jenrette.[4] No difficulty was encountered in repeating the procedure, but the pigmented pseudoglobulin could be deposited almost completely by centrifugation for 15 minutes at 970x gravity. Under the phase contrast microscope the preparation appeared to consist of pigmented particles,

much of which was held together in clumps by background material of low refractive index. Nuclei and an occasional whole cell were also seen. Under the electron microscope the pigmented particles proved to be granules of the same size range and shape as those obtained from the S-91 melanoma by the differential centrifugation procedure given above.

Discussion. Melanin granules are characterized with optical microscopes the resolving power of which—approximately 0.2 μ—is of the same order of magnitude as the granules themselves.[7] The electron microscope has a resolving power of the order of 0.005 μ and is, therefore, the instrument of choice for investigation of granule form. However, it does not reveal the presence of pigment as such. We have therefore established the identity of the micrographed particles with melanin granules by optical means. This identity was, in addition, indicated by the relative absence of the typical pigmented forms in granule preparations from the amelanotic melanoma.

The regular and rounded shapes of a representative group of melanin granules now observed with the electron microscope establish these particles as formed elements. The alternative that they consist simply of precipitated aggregates of a metabolic end-product is obviated; melanin is evidently deposited according to a pattern. The demonstration that melanin granules unchanged in shape or size also make up the melanopseudoglobulin obtained from transplantable mouse melanomas shows that these granules may aggregate in the presence of ammonium sulfate and be redispersed in a measure upon dialytic removal of the salt.

Discontinuities in the micrographic images of melanin granules would give some indication of gross structure. The present series of micrographs however are uniformly dense across the images and conclusions with respect to internal structure cannot be drawn.

Summary. 1. Melanin granules were obtained by differential centrifugation from the S-91 and Harding-Passey transplantable mouse melanomas, from the choroid, ciliary

7 Russell, E., *Genetics*, 1946, **31**, 327.

body and iris of the beef eye, and from colored human skin. These preparations were characterized with the electron microscope.

2. The melanin-containing pseudoglobulin from the S-91 melanoma was shown to consist of melanin granules unchanged in shape or size with respect to granules obtained by centrifugation.

16114

Evaluation of a New Test for the Effect of Vitamin P on Capillaries.[*]

R. J. RAIMAN AND H. NECHELES.

From the Department of Gastro-Intestinal Research, Research Institute of Michael Reese Hospital, Chicago, Ill.

In our work with rutin[1] we felt the need for a quick test for vitamin P like effects on the capillaries. The method described by Majovski *et al.*[2] appeared to have the advantage of speed and simplicity necessary for the assay of a series of compounds.

The test proposed by Majovski *et al.*[2] consists of the following procedures: Control and test mice are placed in a small jar which is connected by means of a stop-cock and pressure tubing to a five-gallon bottle. The larger bottle is evacuated by means of a vacuum pump to 45 mm of mercury pressure. Upon opening the stop-cock, the pressure in the smaller jar containing the mice quickly drops to 70 mm of mercury pressure. After a 60-second exposure to this decreased pressure the mice are removed and observations are made on the extent of pulmonary hemorrhage. Control mice exhibited a greater degree of pulmonary hemorrhage than did those "protected" by prior administration of certain citrus peel fractions. Thus, it was assumed that this method affords a means of measuring changes in vascular fragility or permeability as influenced by various agents.

In the report of this method, the authors made mention of control animals which failed to conform to the usual reaction and indicated that there seemed to be some correlation between the "presence or absence of food in the stomach and these peculiarities." This observation was repeated by Kibrick and Goldforb[3] who reported "protection" afforded by starving the animals prior to their use in the test as compared with fed mice. Besides, Kibrick and Goldforb[3] found that a number of preparations of the hesperidin group did not afford protection to the mice against pulmonary hemorrhage. They admit, however, that the drugs used may have had varying and unknown contents of vitamin P activity. We felt that the observations of Kibrick and Goldforb did not obviate the usefulness of this method as a test for vitamin P activity, as feeding and fasting of the mice were factors easily controlled.

A few preliminary trials with two groups of fed mice, one of which had received rutin, showed that the animals which had been fed only exhibited gross pulmonary hemorrhages, while those which had been fed and which also had received prior treatment with rutin, showed no pulmonary hemorrhages. Thus it appeared that control of the feeding-fasting factor retained the method's capacity for testing for vitamin P activity.

We have found, however, in the course of our work, that the method is not specific for

1 Raiman, R. J., Later, E. R., and Necheles, H., *Science*, in press.

2 Majovski, G. J., Lesser, A. J., Hanson, H. C., Carno, H. O., and Thienes, C. H., *J. Pharm. and Exp. Therap.*, 1944, 80, 1.

3 Kibrick, A. C., and Goldforb, A. F., *J. Pharm. and Exp. Therap.*, 1944, 82, 211.

phase microscope the preparations appeared homogeneous with respect to melanin granules. In the electron microscope the granules varied from circular to elliptical shape, 0.30 μ to 2.00 μ across; they were sharply defined but again failed to reveal internal structure (Fig. 5, 6).

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Summary. 1. Melanin granules were obtained by differential centrifugation from the S-91 and Harding-Passey transplantable mouse melanomas, from the choroid, ciliary

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16114

Evaluation of a New Test for the Effect of Vitamin P on Capillaries.[*]

R. J. RAIMAN AND H. NECHELES.

From the Department of Gastro-Intestinal Research, Research Institute of Michael Reese Hospital, Chicago, Ill.

In our work with rutin[1] we felt the need for a quick test for vitamin P like effects on the capillaries. The method described by Majovski et al.[2] appeared to have the advantage of speed and simplicity necessary for the assay of a series of compounds.

The test proposed by Majovski et al.[2] consists of the following procedures: Control and test mice are placed in a small jar which is connected by means of a stop-cock and pressure tubing to a five-gallon bottle. The larger bottle is evacuated by means of a vacuum pump to 45 mm of mercury pressure. Upon opening the stop-cock, the pressure in the smaller jar containing the mice quickly drops to 70 mm of mercury pressure. After a 60-second exposure to this decreased pressure the mice are removed and observations are made on the extent of pulmonary hemorrhage. Control mice exhibited a greater degree of pulmonary hemorrhage than did those "protected" by prior administration of certain citrus peel fractions. Thus, it was assumed that this method affords a means of measuring changes in vascular fragility or permeability as influenced by various agents.

In the report of this method, the authors made mention of control animals which failed to conform to the usual reaction and indicated that there seemed to be some correlation between the "presence or absence of food in the stomach and these peculiarities." This observation was repeated by Kibrick and Goldforb[3] who reported "protection" afforded by starving the animals prior to their use in the test as compared with fed mice. Besides, Kibrick and Goldforb[3] found that a number of preparations of the hesperidin group did not afford protection to the mice against pulmonary hemorrhage. They admit, however, that the drugs used may have had varying and unknown contents of vitamin P activity. We felt that the observations of Kibrick and Goldforb did not obviate the usefulness of this method as a test for vitamin P activity, as feeding and fasting of the mice were factors easily controlled.

A few preliminary trials with two groups of fed mice, one of which had received rutin, showed that the animals which had been fed only exhibited gross pulmonary hemorrhages, while those which had been fed and which also had received prior treatment with rutin, showed no pulmonary hemorrhages. Thus it appeared that control of the feeding-fasting factor retained the method's capacity for testing for vitamin P activity.

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1 Raiman, R. J., Later, E. R., and Necheles, H., Science, in press.

2 Majovski, G. J., Lesser, A. J., Hanson, H. C., Carne, H. O., and Thienes, C. H., J. Pharm. and Exp. Therap., 1944, 80, 1.

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[7] Russell, E., *Genetics*, 1946, **31**, 327.

16115

Effect on Cardiac Glycogen of Intravenously Administered Sodium Acetoacetate, Sodium Beta-Hydroxybutyrate and Sodium Butyrate.

ROBERT W. LACKEY, CARL A. BUNDE, AND LEROY C. HARRIS.

From the Department of Physiology and Pharmacology, Southwestern Medical College, Dallas, Texas.

Following pancreatectomy[1,2] or in alloxan diabetes,[3] the glycogen content of the liver and skeletal muscles is rapidly depleted, but the glycogen content of the heart is increased. In some recent experiments,[4] we have shown that in rats the amount of glycogen stored by the myocardium parallels changes in the blood ketone level induced by dietary means. In general, the glycogen stores of the liver and skeletal muscles tend to vary inversely with those of the heart. These observations suggested that ketosis may be a causative factor in increasing the deposition of cardiac glycogen, though decreased insulin availability may be important. With the idea of minimizing changes in insulin activity, experiments of shorter duration were carried out.

Method. Two groups of experiments were done using adult male white rats of the Sprague-Dawley strain. The animals of the first group (Table I) were fasted for 24 hours; those of the second group (Table II) were unfasted. Each group was divided into several series, one serving as a control series, the others being subjected to intravenous infusion of 0.9% sodium chloride, 3% sodium chloride, 10% sodium butyrate, 10% dl-betahydroxybutyrate or 10% sodium acetoacetate solution. The solutions were adjusted to a pH of 7.2; the volume infused varied from 5 to 7½ ml; the duration of the infusion was 4 hours; and the time of sacrifice was one hour after termination of the infusion. The animals were anaesthetized with sodium

pentobarbital and the trachea was cannulated routinely except in the uninfused control series of the first group, which was anaesthetized and sacrificed at the time the infusions were begun in the other series of the group.

Since the temperature regulating mechanism in the rat was greatly affected by the anesthetic, heat was applied in an attempt to keep the temperature of each individual animal near the normal level. At a room temperature below 30 to 31°C, supplementary heat from lamps was generally necessary.

At the time of the sacrifice, blood samples were obtained for glucose and ketone body determinations and samples of heart, liver and skeletal muscle were removed for glycogen determination. The various analyses were carried out by methods described earlier.[3]

Results and Discussion. Table I shows that in the fasted series receiving infusions of sodium dl-β-hydroxybutyrate and sodium butyrate, the cardiac glycogen stores were 654 ± 27 and 653 ± 34 mg % respectively, significantly above the values of 502 ± 26 and 510 ± 16 mg % found in the fasted series receiving respectively no treatment or an infusion of 0.9% sodium chloride solution.

The glycogen stores in the skeletal muscle were essentially the same in the experimental and the control series. The liver glycogen levels in the series receiving saline (532 ± 63 mg %) were significantly higher than in the untreated series (183 ± 32 mg %) or the series receiving sodium dl-β-hydroxybutyrate (112 ± 48 mg %). The very great standard error for values obtained for liver glycogen after sodium butyrate infusion makes it impossible to attach any significance to the data on liver glycogen in this series.

The only remarkable finding in blood ketone values was the low level of ketones in the series receiving the saline solution. In

1 Cruickshank, E. W. H., *J. Physiol.*, 1913, 47, 1.

2 Fisher, N. F., and Lackey, R. W., *Am. J. Physiol.*, 1925, 72, 43.

3 Lackey, Robert W., Bunde, Carl A., Gill, A. J., and Harris, Leroy C., PROC. SOC. EXP. BIOL. AND MED., 1944, 57, 191.

4 Lackey, Robert W., Bunde, Carl A., and Harris, Leroy C., *Am. J. Physiol.*, 1946, 145, 470.

TABLE I.

Agent	Amt of drug intraperitoneally	Protection	No. of mice
Simultaneous controls of all tests	Untreated	No	48
Acetate buffer, pH = 7.75	0.15 cc	,,	3
Rutin in acetate buffer, pH = 7.75	1 mg in 0.15 cc	Yes	4
Propylene glycol	0.15 cc	,,	6
Rutin in propylene glycol	1 mg in 0.15 cc	,,	12
Ethylene glycol	0.15 cc	,,	4
Glycerol	,, ,,	No	3
Ethyl alcohol	,, ,,	Yes	4
Oxalic acid	2 mg in 0.15 cc	No	3
Hypertonic saline	0.15 cc	*	5
Glucose	2 mg in 0.15 cc	Yes	4

* Questionable.

vitamin P and are reporting our results here in order to save other workers time and labor.

Observations. On running control mice which received as prior treatment only the fluids used in dissolving the agents which we desired to test for their effects on vascular permeability and fragility, we found that some solvents by themselves afforded equal protection against the hemorrhages induced by exposure of the animals to decreased pressure as the specific agents believed to decrease vascular fragility and permeability. Therefore, we tried the effect of several other agents. Table I lists the agents tested and their ability to protect adult white mice of 20-25 g body weight and of both sexes against the pulmonary hemorrhages. No effect of sex was noted in the tests.

Our method deviated from that of Majovski *et al.* only in that the pressure to which the animals were subjected was slightly higher. We used a pressure of 85 mm instead of 70 mm of mercury.

No apparent pulmonary hemorrhages in treated mice as compared with grossly hemorrhagic lungs of untreated controls simultaneously exposed to the decreased pressure was called "protection," and similar degrees of hemorrhage in control and test animals was declared "no protection."

All agents were administered by intraperitoneal injection 35 minutes before exposure to the decreased pressure.

It might be mentioned that most of the "protected" animals survived significantly longer in the decreased pressure and afterwards than did the controls, but the correlation between survival time and protection is not absolute.

Hypertonic saline afforded "questionable" protection because complete protection was never achieved, but in several trials, the mice given hypertonic saline exhibited significantly less hemorrhage than did the controls.

Except rutin, the agents which were found to protect the animals by this method are generally accepted to have no effect on vascular fragility or permeability. Most of the solvents used for rutin afforded as much protection as did rutin itself. Also glucose, which is an integral part of the molecule of both hesperidin and rutin, afforded protection against the pulmonary hemorrhages.

We conclude therefore, that this test is non-specific for the vascular effects of vitamin P.

Summary. The test proposed by Majovski *et al.* for evaluating the effects of vitamin P on capillaries was subjected to a critical study.

The experience of others that previous feeding affects the results of the test was confirmed, but this objection can be overcome by using starved mice only.

The present tests demonstrate that Majovski's method is non-specific, however, because a number of solvents used for the rutin, as well as other substances, gave mice the same protection against low atmospheric pressure as rutin did. The drugs which protected mice are not known to have any effect on capillaries; they were: propylene glycol, ethylene glycol, ethyl alcohol and glucose.

has been discussed by McQuarrie[7] and Lewis, McKee, and Longwell.[8]

Table II records data from unfasted animals. The cardiac glycogen levels of 681 ± 42 mg % and 724 ± 45 mg % found in the experimental series receiving sodium β-hydroxybutyrate and sodium acetoacetate, respectively, were the highest observed. These series had also the lowest liver glycogen stores (807 ± 251 and 1051 ± 254 mg % respectively) and the highest blood ketone levels; the muscle glycogen levels (566 ± 37 and 614 ± 52 mg %) differed little from those found in the control series receiving the hypertonic and the isotonic saline solution (551 ± 20 mg % and 604 ± 16 mg % respectively). Thus the same parallelism is observed between the occurrence of ketonemia and increased cardiac glycogen stores in this as in the fasted series of animals.

Quite unexpectedly, the non-fasted animals receiving isotonic or hypertonic sodium chloride solution showed higher values for cardiac glycogen, skeletal muscle glycogen, blood ketones and blood sugar than did animals subjected only to dummy operations. These effects were contrary to those observed after similar saline infusion in fasted animals. The meaning of this difference is not apparent to us. The increase in blood sugar observed in the series receiving sodium acetoacetate raises the same question of inter-relationship with cardiac glycogen storage as that discussed in connection with the fasted series receiving sodium butyrate.

No attempt was made in these studies to control the factor of alkalosis which must have followed our administration of the sodium salts of acids capable of being metabolized. Deuel and coworkers[9] have concluded that alkalosis is inimical to hepatic glycogenesis and this may explain the lowering of liver glycogen in certain of our series. Long and Evans[10] observed a lowered cardiac glycogen in rats with pronounced alkalosis, induced by giving them sodium bicarbonate. In our animals cardiac glycogen increased in spite of this adverse condition.

The data presented seem to give some additional support to the idea that there exists a parallelism between blood ketone levels and the deposition of glycogen in the myocardium. Since an artificially produced ketonemia may increase the glycogen stores in the heart within a few hours, either with or without a preliminary fast of short duration, the relationship between ketonemia and cardiac glycogen storage may well be a direct one rather than one indirectly attributable to the decreased insulin availability resulting from conditions giving rise to endogenous ketonemia.

Summary. In experiments carried out on adult male white rats under sodium pentobarbital anaesthesia, intravenous administration of ketone bodies resulted in an increase in the glycogen stores of the heart. Concomitant increase in the glycogen stores of the liver and skeletal muscle did not occur.

7 McQuarrie, Irvine, *Essays in Biology,* p. 413, University of California Press, Berkeley and Los Angeles, 1943.

8 Lewis, Robert C., Jr., McKee, Frances S., and Longwell, Bernard B., *J. Nutrition,* 1944, **27**, 11.

9 Deuel, Harry J., Jr., Butts, Joseph S., Blunden, Harry, Cutler, Charles H., and Knott, Leslie, *J. Biol. Chem.,* 1937, **117**, 119.

10 Long, C. N. H., and Evans, G. T., Proc. Soc. Exp. Biol. and Med., 1932, **30**, 186.

TABLE I.

Tissue Glycogen, Blood Ketones, and Blood Sugar in 24-Hour Fasted Rats Given Sodium dl-β-Hydroxybutyrate and Sodium Butyrate by Intravenous Infusion.

| Solution infused | No. of animals | Glycogen* mg per 100 g tissue | | | Blood ketones mg per 100 ml‡ | Blood sugar mg per 100 ml |
		Liver	Heart	Skeletal muscle		
None	17	183 ± 32†	502 ± 26†	396 ± 28†	17.6	115
Isotonic saline	12	532 ± 63	510 ± 16	385 ± 22	4.6	85
Sodium β-hydroxybutyrate	9	112 ± 48	654 ± 27	421 ± 15	54	111
Sodium butyrate	13	464 ± 264	653 ± 34	362 ± 54	15.8	139

* As glucose.
† Standard error.
‡ As hydroxybutyric acid.

TABLE II.

Tissue Glycogen, Blood Ketones, and Blood Sugar in Non-Fasted Rats Given Sodium dl-β-Hydroxybutyrate and Sodium Acetoacetate by Intravenous Infusion.

| Nature of treatment or solution infused | No. of animals | Glycogen* mg per 100 g tissue | | | Blood ketones mg per 100 ml‡ | Blood sugar mg per 100 ml |
		Liver	Heart	Skeletal muscle		
Untreated	10	1660 ± 200†	451 ± 34†	434 ± 15†	3.2	129
Dummy operation	16	2368 ± 276	510 ± 17	504 ± 24	3.4	116
Isotonic saline	11	3381 ± 309	615 ± 27	604 ± 16	7.6	134
Hypertonic saline	11	2537 ± 330	627 ± 27	551 ± 20	8.5	127
Sodium β-hydroxybutyrate	12	807 ± 251	681 ± 42	566 ± 37	22	136
Sodium acetoacetate	11	1051 ± 254	724 ± 45	614 ± 52	17.3	159

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this the blood sugar level was also lower. Increases in the blood sugar level followed administration of sodium butyrate but not administration of β-hydroxybutyrate,

The rather large increase in cardiac glycogen in the series receiving sodium butyrate and sodium dl-β-hydroxybutyrate, without a concomitant increase in glycogen deposition in either the liver or the skeletal muscle, supports our previously stated concept of a relationship between ketonemia and increased glycogen in the myocardium. The increase in blood sugar level after the administration of sodium butyrate has been observed before[4,5] and may be related to the increased cardiac glycogen; but observations that the elevation of the blood sugar level was not great and that the muscle glycogen stores were not increased suggest that the underlying factors which selectively increased the cardiac glycogen in this case were the same as those

in the ketosis of prolonged fasting, which is not accompanied by an increase in blood sugar level.[1] In the fasted series in which dl-β-hydroxybutyrate was given, the increased cardiac glycogen was not associated with an increase in blood sugar.

In the fasted series infused with saline solution there was no change in the skeletal muscle, or cardiac glycogen. The increase observed in the liver glycogen may have been attributable to the decrease in muscular activity incident to anesthesia or to the fact that in fasting rats the liver glycogen stores are depleted in the early stages of the fast, but tend to be replenished as the fast is prolonged.[6] The pronounced lowering of blood ketones and blood sugar in this series is difficult to explain, but may be related to the effect of sodium chloride on the insulin producing mechanism or the anti-insulin or anti-ketogenic mechanism, an action which

5 Markees, Silvio, Klin. Wchnschr., 1941, 20, 1260.

6 Bunde, Carl A., and Lackey, Robert W., unpublished data.
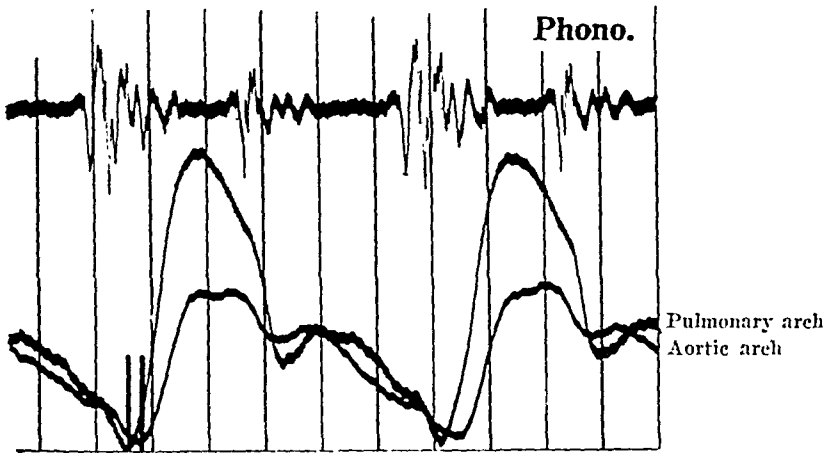
FIG. 1.
Simultaneous fluorocardiograms of the pulmonary and aortic arches. Phonocardiogram.
The marks indicate the beginning of systolic expansion of the arteries.
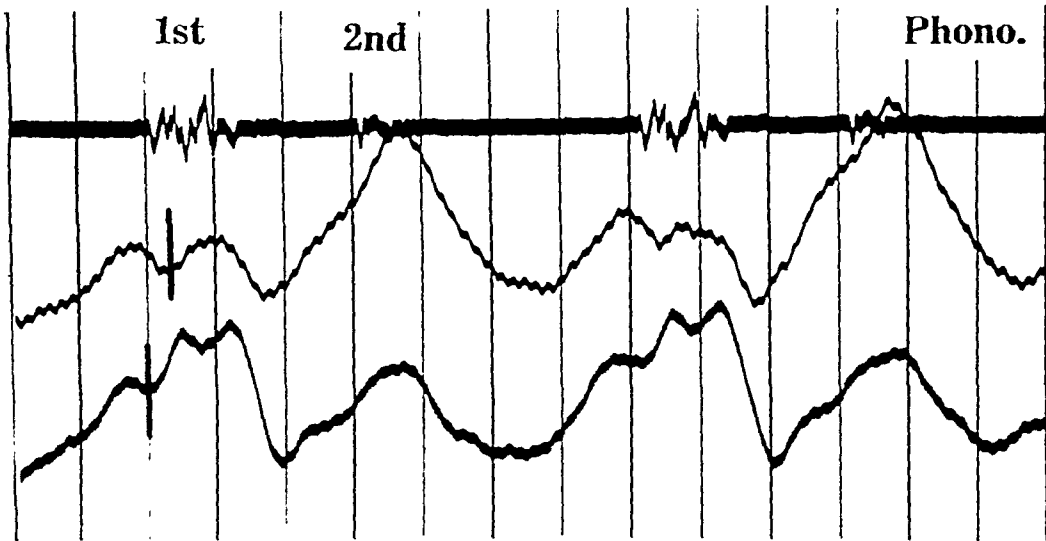
FIG. 2.
Simultaneous fluorocardiograms of the right (bottom) and left (middle) auricles. Phonocardiogram.
The marks indicate the maximum depth of the auricular contraction.
The other waves bear a similar temporal relationship.

the activities of the right and left heart presented new problems, which were solved in the following way: (a) The study of the pulsations of the pulmonary arch and of the aortic arch was made with the subject in the postero-anterior position by applying the slits of the 2 pickup units across the borders of the curves corresponding to the 2 large vessels. (b) The study of the pulsations of the 2 auricles was made with the subject in the right oblique position by applying the slits, one below the right bronchus (left auricle), the other much lower (right auricle). (c) The study of the pulsations of the 2 ventricles was made in the left lateral position applying one slit as low as possible across the anterior border of the cardiac shadow (right ventricle) and the other across the lower posterior

7 Luisada, A. A., Fleischner, F. G., and Rappaport, M. B., *Am. Heart J.*, in press, 1948. Note II.

16116

Temporal Relation Between Contraction of Right and Left Sides of the Normal Human Heart.

Aldo A. Luisada and Felix G. Fleischner.

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Knowledge of the normal temporal relationship of contraction of the two auricles and ventricles is important in various clinical conditions. Only indirect or semi-direct evidence of the activities of the various cardiac chambers has been available so far in clinical cases. As a new method permits their direct observation, a study of normal subjects has been undertaken, as a preliminary to clinical studies.

Precedents. On the basis of simultaneously recorded right ventricular pressures, carotid surface pulses and ballistocardiographic recoils (direct method for the right ventricle only), Hamilton and co-workers[1] found an asynchronous ejection of the two ventricles in man. Either ventricle may precede the other.

The only available study on man with similar technique was briefly reported by Chamberlain and co-workers.[2] These authors studied the ascending aorta and the first part of the pulmonary artery by means of the electrokymograph. The study was made either with a single pickup device and subsequent tracings or with 2 separate pickups and 2 galvanometers. In the latter instance two simultaneous separate tracings were recorded, permitting comparison. These authors found a variable time relationship of the pulses of the 2 vessels; either ventricle might precede the other; in a few cases the pulses were simultaneous.

Method. The method used in this study is based on the combination of the fluoroscope, a photoelectric pickup unit, a screen with a slit and an electrocardiograph. The apparatus,

originally described by Henny, Boone and Chamberlain[3,4] and called "electrokymograph" was subsequently modified by us together with Rappaport[5,6] and the name "fluorocardiography" was proposed, as being more descriptive of the method.

The pickup unit is adjusted under fluoroscopic control so that the motion of a small section of the margin of the cardiac silhouette is seen within the slit. Any outward motion is recorded as a positive wave on the tracing; any inward motion, as a negative wave. Identification of these waves is possible by the simultaneous recording of a tracing of the heart sounds. The recorded tracings are similar to pulse tracings (arteries) and to volume tracings of the ventricles or auricles. Therefore, no new names are necessary for the waves, these being the same as in sphygmograms or cardiograms.

It is to be noted that the motions of the ventricular borders are somewhat less typical when recorded in the lateral positions even if the beginning and end of the various waves bears the normal temporal relation to the heart sounds.

Two Sanborn apparatus for fluorocardiography and a Sanborn Tri-beam Stethocardiette were used for the study. Observations were made on 8 normal subjects between the ages of 14 and 46.

The general method previously described by us for the positioning of the slit[5,6,7] was followed. However, the simultaneous recording of

1 Hamilton, W. F., Attyah, A. M., Remington, J. W., Wheeler, N. C., and Witham, A. C., Proc. Soc. Exp. Biol. and Med., 1947, 65, 266.

2 Chamberlain, W. E., Boone, B. R., Ellinger, G. F., Henny, G. C., and Oppenheimer, M. J., *Fed. Proc.*, 1947, 6, 88.

3 Henny, G. C., and Boone, B. R., Am. J. Roentgen., 1945, 54, 217.

4 Henny, G. C., Boone, B. R., and Chamberlain, W. E., Am. J. Roentgen., 1947, 57, 409.

5 Luisada, A. A., Fleischner, F. G., and Rappaport, M. B., New England Heart Assn., Feb. 24, 1947.

6 Luisada, A. A. Fleischner, F. G., and Rappaport, M. B., Am. Heart J., in press, 1948. Note 1.
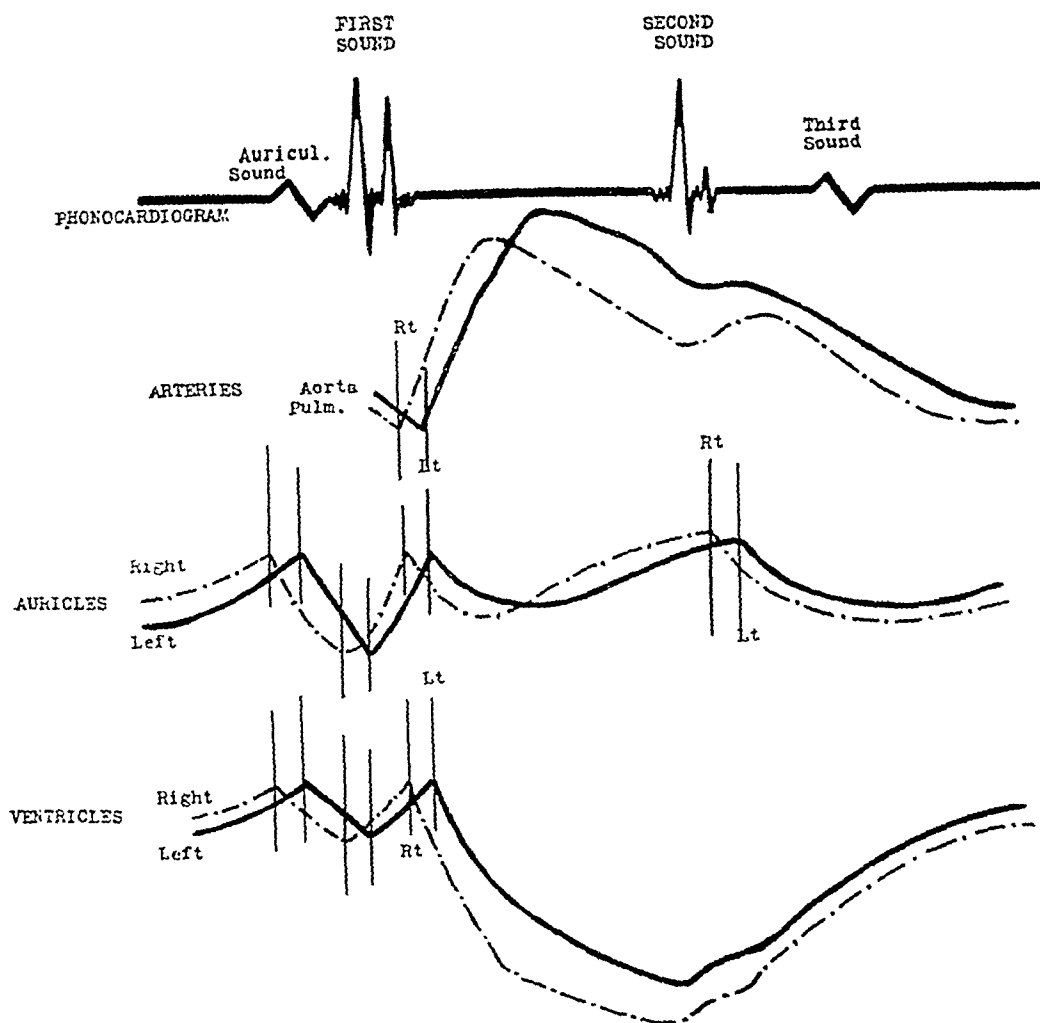
FIG. 4.

Temporal relation between contraction of right and left sides of the normal human heart. Schemes made from tracings of the various subjects.

the right ventricle. (b) The pulsation of the aortic arch takes place some time after the beginning of the ejection of the left ventricle. (c) The speed of the pulse wave is lower in the lesser than in the greater circulation, as indicated by Hamilton[9] and confirmed by us.[7] Therefore, in spite of the different length of the 2 large vessels, it is understandable that

the time relationship between their pulses turns out to be about the same as that between the beginning of ejection of the 2 ventricles. We can conclude that all data point to the fact that under normal conditions the right ventricular contraction precedes the left. This fact is in accord with the demonstration that, while the left side of the septum is excited before the right, the external wall of the left ventricle is reached after that of the right (Lewis[10]). The time

8 Chavez, L., Dorbecker, N., and Celis, A., Am. Heart J., 1947, 33, 560.

9 Hamilton, W. F., in Howell's Textbook of Physiology by John Fulton, Philadelphia, Saunders, 1946, p. 541.

10 Lewis, T., The Mechanism and Graphic Registration of the Heart Beat, London, Shaw, 1925.
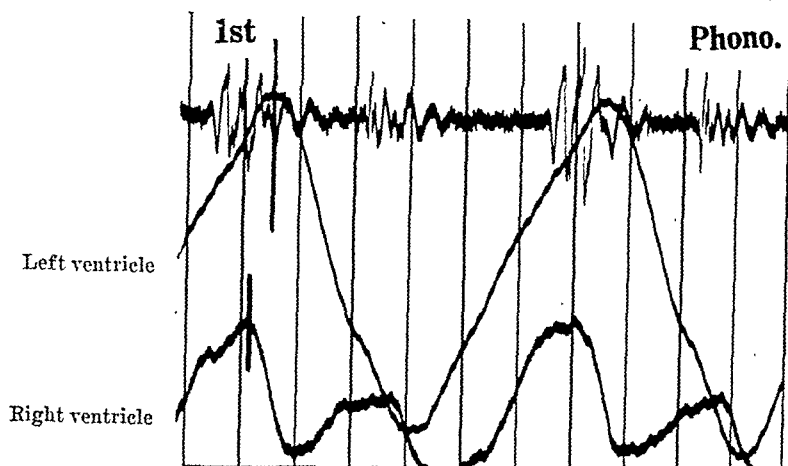
FIG. 3.
Simultaneous fluorocardiograms of the right and left ventricles. Phonocardiogram.
The marks indicate the beginning of ventricular ejection.

border (left ventricle).

Results. (1) The simultaneous tracings of the pulsations of the pulmonary artery and the aortic arch (Fig. 1) show the following time relationship: (a) the rise of the pulse of the pulmonary artery precedes that of the aorta by 0.020 to 0.025 seconds (20 to 25 msc). (b) The peak of the pulse of the pulmonary artery precedes that of the aortic pulse by about 0.03 seconds (30 msc).

(2) The simultaneous tracings of the pulsations of the 2 auricles (Fig. 2) show the following time relationship: (a) The beginning of the auricular contractions is not always apparent owing to the rotation of the patient. When visible, the right auricle precedes the left. (b) The maximum depth of contraction is reached by the right auricle about 0.025 seconds (25 msc) before the left. (c) The maximum dilatation of the auricle, at the moment in which the auriculoventricular valve opens, is reached by the right auricle from 0.025 to 0.030 seconds (25 to 30 msc) before the left.

(3) The simultaneous tracings of the pulsations of the anterior and posterior aspects of the heart, namely of the 2 ventricles (Fig. 3) show that these bear the following relationship: (a) The beginning of ventricular contraction (isometric contraction) cannot be accurately ascertained in the lateral posi-

tion. (b) The beginning of the ejection period is indicated by a sharp fall of the tracing after the maximum has been reached subsequent to auricular contraction. This occurs about 0.025 seconds (25 msc.) earlier for the right ventricle (anterior surface) than for the left ventricle (posterior surface).

Temporal relation between contraction of right and left sides of the heart are further shown in the schemes of Fig. 4.

Comment. These observations concerning the temporal relations in cardiac action confirm each other. Actually, the final conclusions concerning the time relationship of the ventricular pulsations are based on the correlation of tracings obtained from the auricles indicating the opening of the A-V-valves; from the ventricles showing the beginning of ejection from both ventricles; and from the large vessels revealing the beginning rise of the aortic and pulmonic pulses. The evidence indicates that the right ventricle contracts first, and the interval between the contractions of the 2 ventricles is between 0.025 and 0.030 seconds (25 to 30 msc). The same interval exists between the pulmonic and aortic pulses. The latter is due to various facts: (a) the pulsation of the middle arch is actually that of the left branch of the pulmonary artery,[8] taking place, therefore, somewhat after the beginning of ejection of

were administered daily for more than a week.

A search for other carcinoclastic microbial products was then undertaken. For a survey of a large number of micro-organisms, *in vivo* test is too slow and interpretation is likely to be uncertain. Accordingly the test chosen was the histological condition of surviving tissue slices, as used in metabolism studies, after incubation for 24 hours with the bacterial preparation under investigation. A slice of the liver of the same animal was used as an indicator of the selectivity of the preparation for tumor or normal tissue. Penicillin, it was found, could be used to exclude extraneous micro-organisms.

Materials and Methods. Webster strain mice bearing spontaneous carcinomata, and pigmented rabbits carrying the transplantable Brown-Pearce carcinoma supplied the tumor and liver slices used in these experiments. Tissues which showed extensive necrosis or were otherwise unusual were discarded.

Mice were killed by a blow on the head and rabbits by injecting air into a marginal vein. The tumor and liver were removed, rinsed in physiological saline solution, and the tissues sliced into sections not more than 0.3 mm thick, either with a straight razor or a Stadie[4] microtome. Mouse liver was generally cut by hand, while mouse tumor and rabbit liver and tumor were more conveniently cut on the microtome. The slices were rinsed through 3 baths of physiological saline solution before being placed in 20 ml beakers for incubation. Each beaker contained 4 ml of the medium, a slice of tumor, and as a control tissue, a slice of the autologous liver.

All procedures were carried out as aseptically as possible.

Previous tests showed that tissues incubated in serum under conditions later described preserved a more nearly normal gross and microscopic aspect than they did when incubated in balanced salt solution. Heterologous serum appeared to be as favorable as the homologous serum, consequently the basic medium consisted of rabbit serum ob-

tained from blood collected by aseptic cardiac puncture to which 100 mg of dextrose and 100,000 units of penicillin (Squibb) were added per 100 ml of serum before use. The usual procedure employed 3.5 ml of serum containing sugar and penicillin plus 0.5 ml of the culture or preparation being tested.

A shaker-incubator[5] equipped to hold thirty 20 ml beakers allowed the agitation to be carried out at any desired speed, temperature, and under any preferred water-saturated gas mixture. Tissues in these experiments were incubated for 24 hours at 37°C under 95% oxygen and 5% carbon dioxide and shaken at 80 cycles per minute.

All preparations were run in duplicate, and with each set of experimental beakers a pair of beakers containing only serum and the tissue slices were incubated as control. In experiments not involving the use of penicillin in all beakers, an additional control pair without penicillin was run.

Specimens of tumor and liver were routinely fixed in Bouin's or Carnoy's fluid at the beginning of each experiment, and at the conclusion, all tissues were immediately fixed in preparation for microscopic examination.

The slices were sectioned at 8 μ in a plane parallel to the original slicing to give large area for examination. Orientation was assured by sandwiching the slices between sheets of agar gel prior to fixation.[6] Hematoxylon-eosin was the routine stain.

The basic medium for growing the bacteria was as follows:

Yeast extract (Difco)	3 g
Peptone (Difco)	3 "
Dextrose	5 "
Tapwater	1 L

Adjusted to pH 8.0 with NaOH prior to autoclaving.

Most of the organisms tested grew well in this medium at room temperature. For the culture of *Sporosarcina ureae* and other urea decomposing bacteria 20 g urea was added to the above medium.

Results. In a general survey of micro-organisms whose products might selectively

4 Stadie, W. C., and Riggs, B. C., *J. Biol. Chem.*, 1944, 154, 687.

5 Dubnoff, J. W., in preparation.

6 Cohen, A. L., in preparation.

interval between excitation of the right and left ventricles was found by Lewis to be only 0.01 seconds. As we found it, the interval was greater; this may be attributed to the fact that we measure the interval between the beginning of the ejection from the two chambers. We know that in the human heart the isometric contraction of the right ventricle lasts 0.01 sec. (Richards and co-workers[11]) but, we do not yet know the duration of the isometric contraction of the left. It is likely that the higher pressure existing in the aorta causes a slightly longer duration of the period of tension of the left ventricle, thereby exaggerating the delay already existing.

Our observations on the pulsations of the auricles indicate that, here too, there is a brief interval, the right auricle contracting before the left. Again our observation confirms known physiological facts. In the dog, the sino-auricular impulse reaches the left atrial appendage 0.015 seconds later than the right (Lewis,[10] Wiggers[12]). It is logical

to assume that the interval is longer in the human heart owing to the greater length and difference in length of the muscular bundles leading from the sino-auricular node to either auricle respectively.

The constancy of our findings is in contrast with the variability of results of previous studies.[1,2] This can be explained by the fact that those studies were performed partly with indirect measurements[1] or with a different technique.[2]

Summary. The temporal relation between the contractions of the left and right heart chambers was studied by means of fluoro-cardiography and simultaneous recording of 2 pulse tracings on one strip. The study was performed on 8 normal subjects and included the observation of the pulsations of the aorta and the pulmonary artery, both auricles and both ventricles. In all observations the contraction of the right auricle preceded that of the left auricle and the contraction of the right ventricle that of the left. The delay of action of the left chambers was found to be between 25 and 30 milliseconds.

11 Richards, D. W., Cournand, A., Motley, H. L., Dresdale, D. T., and Ferrer, M. I., *Trans. Assn. Am. Phys.*, 1947, in press.

12 Wiggers, C. J., *Physiology in Health and Disease*, Philadelphia, Lea and Febiger, 1944.

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The Effect of a *Sporosarcina ureae* Preparation on Tumor Cells *in vitro.*[*]

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The report that cell division in sea urchin eggs was inhibited by penicillin[1] was the incentive for these experiments designed to test the effect of microbial products on tumor

cells. Cornman[2] and Lewis[3] found tumor cells in tissue culture damaged or killed by the addition of suitable concentrations of penicillin, though Lewis believed the selective carcinoclastic effect was due to impurities in the preparations.

In experiments not otherwise reported, we injected penicillin into Webster strain mice bearing spontaneous mammary carcinomata. Damage to the tumor in the form of hemorrhage, necrosis, and pyknosis was apparent but only after 50-100,000 units of penicillin

1 Henry, R. J., and Henry, M. D., *J. Gen. Physiol.*, 1945, **28**, 405.

2 Cornman, I., *J. Gen. Physiol.*, 1944, **28**, 113.

3 Lewis, M. R., *Science*, 1944, **100**, 314.

selective carcinoclastic effects.

In an attempt to enhance the effect *Sporosarcina ureae* was grown under the following different conditions:

A. Two-liter flasks containing 500 ml of culture fluid inoculated with *S. ureae* were incubated at 30°C for 48 hours with constant agitation. These heavy cultures harvested at the peak of growth produced a slighter effect than those grown under less favorable cultural conditions. Further experiments indicated that the carcinoclastic effect was greater with cultures which had passed the peak of growth.

B. A 500 ml culture was prepared, agitated at 30°C for 48 hours, and allowed to remain undisturbed at room temperature (ca. 22°C). After 48 hours a sample (BI) was lyophilized, and the remainder (BII) was lyophilized after 120 hours at room temperature. When reconstituted with distilled water sample BI showed little or no carcinoclastic effect, while tumor tissue exposed to sample BII was totally destroyed, the nuclei densely pyknotic, cell boundaries extinguished, and the acinar structure collapsed. There was no perceptible effect upon the liver.

In studies with whole culture, washed bacteria and cell-free medium, it was regularly observed that most pronounced carcinoclasis was obtained either with the washed concentrated cells or with whole old culture.

C. Plates were made of the yeast extract urea medium solidified with a 2% agar, heavily seeded with *S. ureae* and incubated at 30°C for 48 hours. The bacteria were washed off with distilled water, the washings collected and lyophilized. The bacteria from four 200 mm diameter petri dishes were resuspended in 20 ml distilled water after lyophilization, a few drops of methylene blue added as redox indicator, and the suspension divided into four 5 ml lots incubated 18 hours under the following conditions:

C I. Anaerobic at 55°C Methylene blue incompletely reduced.

C II. Anaerobic at 36°C Methylene blue incompletely reduced.

C III. Room temperature under toluene. Methylene blue incompletely reduced.

C IV. 30°C under toluene. Methylene blue reduced in one-half hour. Toluene removed from C III and C IV by vacuum drying, residue reconstituted to original volume.

Preparation C IV, that autolysed under toluene at 30°C, produced complete disintegration of the tumor tissue and did not damage the liver as far as could be seen from stained preparations. Next in order of effectiveness were preparations C I and C II. C III had little effect.

Rabbit Brown-Pearce carcinoma was tested in a similar manner as the Webster strain mice carcinomata (above) and similar results were obtained.

The factor in *Sporosarcina ureae* toxic to tumor and not to liver cells appears to be contained within the bacteria and increases in the medium as these are aged. The evidence for this conclusion is the greater effectiveness of old cultures, the complete and selective destruction of tumor tissue by the bacteria autolyzed at 30°, and the ineffectiveness of the cell-free culture medium in which the bacteria had grown.

Discussion. As far as we are aware, the test method used in this study has not been employed hitherto in the study of or search for substances selectively toxic to tumor cells. It holds out the following advantages: it is rapid, economical, the one animal, even the one tissue, supplies control and experimental specimens, and indirect (immunological and vascular) effects of the test substances are inoperative.

Kidd[8] used a somewhat analogous approach. He found that certain tumor cells after treatment with culture filtrates of *Aspergillus fumigatus* failed to grow after implantation, although there was no visible injury in the tumor cells.

Summary. The incubation of mouse spontaneous carcinoma, rabbit Brown-Pearce carcinoma, and their autologous liver slices with bacterial cultures showed in general that cells of both liver and tumor were uninjured or injured equally. Cultures of *Sporosarcina ureae* damaged tumor cells and left liver cells histologically unaffected. Evidence was obtained that the selective carcinoclastic factor

⁸ Kidd, J. G., *Science*, 1947, **105**, 511.

damage tumor tissue, the variety of growth habits made it necessary to modify the conditions of testing them. Thus bacteria which could grow rapidly in serum at 37°C were added as small inocula; those which might grow to some extent but not sufficiently to swamp the tissues were added either as loopfuls of rich culture to beakers containing 4 ml of serum minus penicillin or as 0.5 ml concentrates of rich cultures to 3.5 ml of serum containing penicillin; while those organisms which could not grow under the conditions described were added as 0.5 ml concentrates to each beaker containing 3.5 ml serum with penicillin added to suppress the growth of contaminants.

Some bacteria damaged both liver and tumor cells, the severity of injury ranging from slight eosinophila and paling of the nuclei to complete disintegration. These were: *Pseudomonas caryocyanea, P. putida, P. sp., Serratia marcescens, Escherichia coli communis, E. aurescens, Shigella sonnei* and *Sarcina sp.*

The majority of micro-organisms tested exhibited no perceptible effect upon either the tumor or liver. In this category are *Pseudomonas indoloxidans, P. ovalis, P. omnivorans, P. acidovorans, P. pyocyanea, P. tumefaciens, Vibrio metchnikovii, Rhodospirillum rubrum, Escherichia coli* (2 strains), *E. coli communior* (compare with *E. coli communis* of preceding list) *E. acidi-lactici, Aerobacter aerogenes, A. cloacae, Alcaligenes fecalis, Salmonella pullorum, Bacterium alboflavus, Proteus vulgaris, Kurthia zopfii, Staph. citreus, Strep. anhemolyticus, Strep. sp., Microccus ureae, M. cinnabareus, Sarcina sp., Bacillus brevis, Aerobacillus polymyxa, Corynebacterium creatinovorans, Mycobacterium phlei, M. salmonicolor, M. sp., Actinomyces coelicolor, Penicillium notatum.*

In addition to these organisms 3 trypanosomes, *Trypanosomum brasiliensis, T. lewisii,* and *Schizotrypanosomum cruzii* were tested because of Roskin's report[7] of their use in producing tumor regression. They were ineffective in our test.

A perceptible specific carcinoclastic effect was produced by preparations of the following

7 Roskin, G., *Cancer Research,* 1946, **6**, 363.

organisms: Bacterium I (an unidentified, slender, motile, non-sporulating rod isolated from contaminated serum), *Spirillum virginianum, Phaeomonas varians, Urobacillus pasteurianum,* and *Sporosarcina ureae.* Of these 5 organisms *Sporosarcina ureae* had the most pronounced differential destructive effect upon tumor tissues. It was selected, therefore, for further study.

After incubation with *Sporosarcina ureae* a slice of spontaneous carcinoma of the Webster mouse underwent the following degenerative changes: The nuclei became pyknotic, the cell membranes shrank and the characteristic acinar structure was largely obliterated. The liver slice cells appeared to be undamaged.

In the first experiments, *Sporosarcina ureae* was grown in 50 ml Erlenmeyer flasks containing 10 ml of culture medium at room temperature. The cultures were harvested at the peak of growth and 0.5 ml of culture was added to each beaker containing 3.5 ml of serum with mouse liver and tumor slices prepared as previously described. Experiments were made with and without the use of penicillin in the concentration of 1000 units per ml. No effect on the action of the *S. ureae* culture being observed, all further experiments were done with penicillin in the medium to safeguard tissues and serum against the growth of contaminants.

Because of the high concentration of ammonia in the *Sporosarcina ureae* cultures addition of 0.5 ml to 3.5 ml of serum gave a pH of 9.5. It was possible, then, that the specific toxicity of the *Sporosarcina ureae* culture to tumor cells resided in either the ammonia present in high concentration or the high pH induced rather than in a more specific toxic substance. To test these possibilities tumor and liver slices were incubated in the serum medium to which either ammonium hydroxide or sodium hydroxide were added to give a pH of 9.5 or higher. No damaging effect of either ammonium or hydroxyl ion at these concentrations was observed on either the tumor or liver cells. Nor did removal of the ammonia from various *Sporosarcina ureae* preparations lessen their
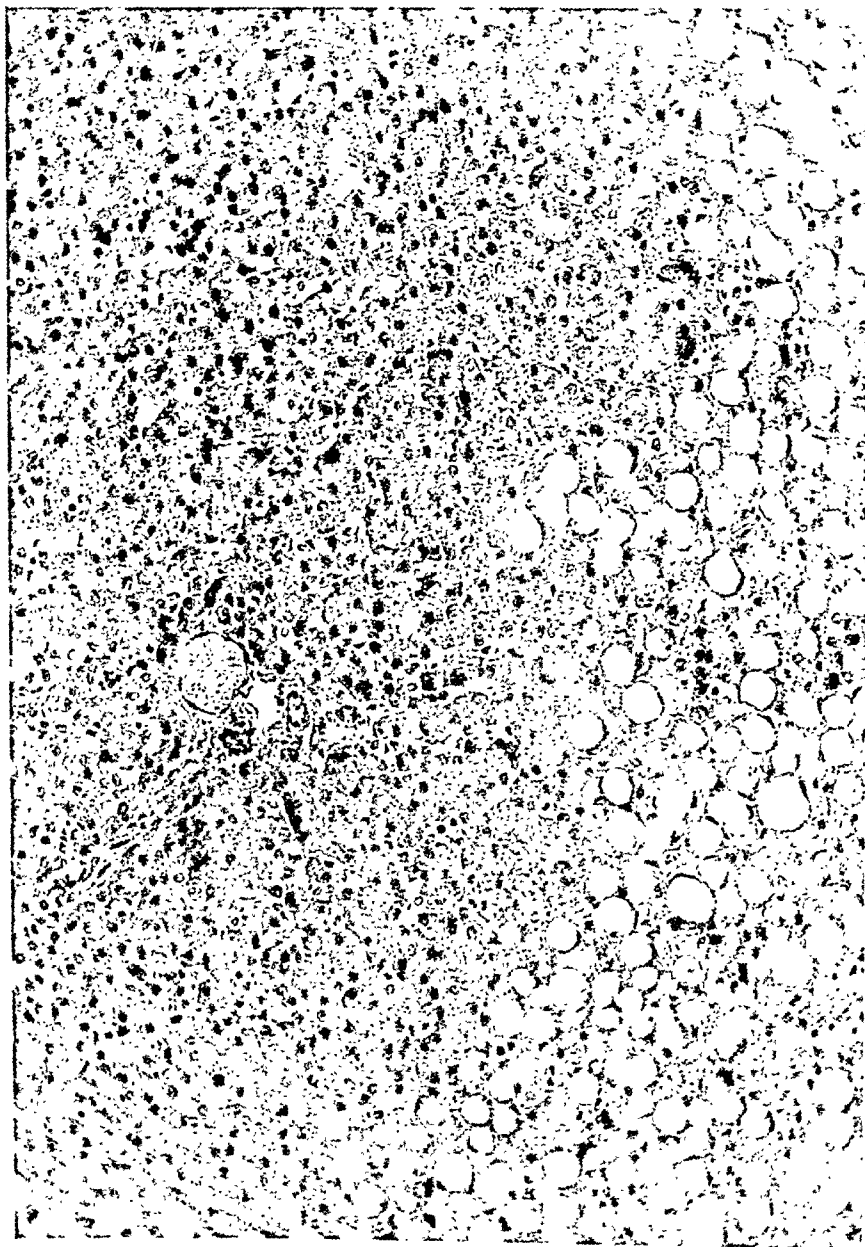
FIG. 1.

Microphotograph, Zeiss × 235, hematoxylin and eosin. The figure is from biopsy material of the liver of animal 3, Group III, on the fourth day of the use of 2,3 dithiopropanol, 30 mg per kilo. Fatty changes indicated by the appearance of larger and smaller droplets of such material are more marked in the periphery of the liver lobule and diminishes as the area of the central vein is approached. In the periphery of the lobule cellular degeneration is marked while in the region of the central vein cell structure is well preserved.

of *S: urcae* cultures was contained within the cells and released on autolysis.

We are indebted to Dr. C. B. van Niel of the Hopkins Marine Station for free access to the culture collection, to Colonel Max Levine for cultures of several of the Enterobacteriaceae, and to Dr. William Negherbon for cultures of trypanosomes and detailed directions for their maintenance.

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Liver Injury in the Dog Following Use of 2-3-Dithiopropinol.* (BAL).

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Fifteen adult dogs furnish the basis for the observations which are to follow. The animals were kept in metabolism cages and given a diet of Purina Dog Chow. No restriction as to the amount was made in the diet or in the water intake. The dithiol was given intramuscularly at 9 a. m. and 9 p. m. on 4 successive days. The dogs were divided into 3 groups. The animals of Group I were given 5 mg per kilo of the dithiol per dose, those of Group II, 15 mg and the members of Group III, 30 mg per kilo. Prior to the commencement of the injections liver function studies were undertaken by the use of bromsulfalein according to the technique devised by Rosenthal and White.[1] For the normal animals and those of Group I, such observations have been satisfactory in that all of the dye was removed from the plasma within half an hour. In the animals of Group II and Group III receiving respectively 15 and 30 mg of dithiol per kilo the rate of removal was invariably prolonged beyond the half-hour period. The percentage removal however during this and subsequent periods was extremely variable. The evidence for the development of a liver injury from the dithiol is not confined to such variable functional observations but has been ascertained by obtaining biopsy material from the livers and by the study of such tissue in those animals that came to autopsy. The tissue was stained for lipoid

material with Scharlach R and also with hematoxylin and eosin. A study of such material permits the following conclusions:

1. In the animals of Group I which received 5 mg of 2-3-dithiopropinol per kilo it was difficult if not impossible to ascertain by such a micro-chemical method whether or not there was any actual increase in lipoid material in the hepatic epithelium over that which can be frequently observed in such tissue designated as normal. In such animals lipoid material appears in the liver in the form of dust-like and larger particles or as minute droplets. This material is more noticeable in the periphery of the lobules following the use of a dithiol.

2. In the animals of Groups II and III which received either 15 or 30 mg of the dithiol per kilo, there develops a marked increase in stainable lipoid in the liver lobules in the form of larger or smaller droplets which may obscure the nuclei of such cells and replace the greater portion of the cell cytoplasm. Such a development is more uniform and more marked in the periphery than in the central portions of the lobules. In general as this area of the lobule is reached the lipoid material is not only reduced in amount but makes its appearance as small discrete droplets. This order of hepatic cell injury is more severe and develops earlier in the animals of Group III that were given 30 mg of the dithiol per kilo. In one animal of this group there developed by the fifth day of the experiment an extensive liver necrosis. The animal became comatose with

1 Rosenthal, S. M., and White, E. C., *J. A. M. A.*, 1925, **84**, 1112.

TABLE I.
Composition of Two Basic Synthetic Diets.
(Parts per cent.)

Diets	I	II
Casein	30	15
Corn starch	59	74
Fat	1	1
Cod liver oil	1	1
Bulk	1	1
Sodium chloride	4	4
Mineral mixture	4	4
Supplements:		
Thiamine chloride	0.8 mg/100 g diet	
Riboflavin	,,	,,
Pyridoxine	,,	,,
Ca pantothenate	4.0	,,
Nicotinic acid	1.0	,,
Choline chloride	100	,,
Tocopherol acetate	10 mg/rat/weekly	

of blood pressure determination gives the mean pressure only.

Kidney lesions. The incidence and severity of the kidney lesions were estimated by a procedure previously described.[3]

Diets. The complete composition of the diets employed is given in Table I.

Experiments. In a first series of experiments each group consisted of 15 rats. The experiment was then repeated with 10 animals in each group. The results were almost identical and will therefore be described conjointly.

Since under certain conditions[1] choline chloride appeared to counteract the hypertensive action of anterior pituitary preparation, an additional group of 10 rats was treated exactly as those on Diet I but they received 10 times the amount of choline chloride indicated in Table I.

Results. Table II summarizes our results. The average hypertension given in Table II refers to the average of the hypertensive animals only. It seemed appropriate to us not to include in the calculation the few normotensive rats, in order to obtain the true hypertensive average.

Confirming previous findings of this laboratory[2] it may be seen in Group 2 that the implantation of DCA produced a marked elevation of the blood pressure. This hypertension, which was present in almost all animals, was completely established on the 20th day of the experiment; it persisted for a period of at least 20 days which elapsed between the two blood pressure determinations. The kidney lesions in this group were also very prominent, as judged by the high incidence and severity of the pathologic changes.

We wish to emphasize particularly that a 15% casein diet does not prevent the production of hypertension and nephrosclerosis under these conditions. In fact our results show that at the end of 40 days 100% of the animals in Group 1 were hypertensive, with an average blood pressure of 161 mm Hg and had as marked kidney lesions as in the 30% casein group.

Ten times the usual amount of choline chloride (Group 3) effected no detectable improvement.

Discussion. Our earlier experiments[1] showed that the hypertension produced by anterior pituitary overdosage is prevented by a 15% casein diet: the present observations indicate that DCA-hypertension, on the other hand, cannot be prevented in this manner. According to the working hypothesis prevalent in this laboratory, the anterior pituitary increases the blood pressure by stimulating the adrenal cortex.[4,5] Since it now appears that the DCA-hypertension is obtained even on low protein diets, it is probable that the site of action of dietary proteins is between the pituitary and adrenal cortex: either proteins increase the efficacy of the corticotrophic hormone or they stimulate corticotrophin production. Experiments on hypophysectomized rats are now under way in this Institute to elucidate this last-mentioned point.

Choline chloride had no influence upon the establishment of hypertension by DCA overdosage.

Summary. While the hypertension and nephrosclerosis produced in the rat by anterior pituitary preparations are prevented by low-

[2] Selye, H., and Pentz, E. I., *Canad. M. A. J.,* 1943, 49, 264.

[3] Hay, E. C., and Seguin, P., *Am. J. Physiol.,* 1946, 147, 299.

[4] Selye, H., and Stone, H., *J. Urology,* 1946, 56, 399.

[5] Hall, C. E., Dontigny, P., Beland, E., and Selye, H., *Endocrinology,* 1946, 38, 296.

an air-hunger type of respiration and died on the fifth day.

3. The experiments indicate the ability of a dithiol when given to normal dogs in an appropriately large amount per kilo to induce an hepatic injury which is characterized by fatty degeneration of the hepatic epithelium and rarely by a necrosis of this tissue. Such observations do not necessarily imply that a similar order of cellular injury would be induced in the dog under the influence of an intoxication by salts of certain of the heavy metals. Such bodies offer a bond of union for the dithiols which in turn may prevent a toxic effect which they are capable of inducing in tissues of normal constitution.

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Influence of Diet upon the Hypertension and Nephrosclerosis Produced by Desoxycorticosterone Acetate Overdosage.*

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We have reported in a previous paper[1] that the hypertension and nephrosclerosis produced by administration of large amounts of anterior pituitary preparations may be prevented by decreasing the protein content of the diet from the normal of about 25% to 15%. In that publication attention was also called to the fact that adrenal enlargement was always marked in the hypertensive animals, which had been kept on a high protein diet, but was much less pronounced in the normotensive ones, which were given a low protein ration.

In order to clarify further the mechanism by which dietary protein affects the production of hormonal hypertension, we have now investigated its role in the production of the hypertension and nephrosclerosis induced by desoxycorticosterone acetate overdosage.

Material and Methods. Production of hypertension. Hooded castrated male rats, weighing 45 to 65 g, were unilaterally nephrectomized and implanted subcutaneously with two 40 mg pellets of desoxycorticosterone acetate (DCA). The animals were then divided into 2 groups: one received a 30% (Diet I), the other a 15% (Diet II) casein ration, both containing a large amount (4%) of NaCl, (for details see Table I). At the end of 40 days the surviving animals were killed and their kidneys and adrenals weighed and sectioned for histologic study, after fixation in "Susa" mixture. Eight animals in Group 1 and 5 rats in Group 2 were killed on the 20th day.

Blood pressure determinations. The blood pressure was determined by carotid cannulation (care being taken to avoid hemorrhages) on a representative number of rats on the 20th day of the experiment and in all surviving animals the day before autopsy, excepting 3 animals in which we missed the determinations. In order to spare the animals no control determinations were performed at the beginning of the experimental period; this appeared permissible as we had previously established[1] that the normal blood pressure of such rats, under our conditions, is 108 ± 14 (standard deviation) mm Hg. Statistical considerations revealed that 108 ± 28 mm Hg would include 95% of all normal rats, hence 135 mm Hg was taken as the highest normal blood pressure. The direct method

TABLE I.
Composition of Two Basic Synthetic Diets.
(Parts per cent.)

Diets	I	II
Casein	30	15
Corn starch	59	74
Fat	1	1
Cod liver oil	1	1
Bulk	1	1
Sodium chloride	4	4
Mineral mixture	4	4

Supplements:		
Thiamine chloride	0.8 mg/100 g diet	
Riboflavin	,,	,,
Pyridoxine	,,	,,
Ca pantothenate	4.0	,,
Nicotinic acid	1.0	,,
Choline chloride	100	,,
Tocopherol acetate	10	mg/rat/weekly

of blood pressure determination gives the mean pressure only.

Kidney lesions. The incidence and severity of the kidney lesions were estimated by a procedure previously described.[3]

Diets. The complete composition of the diets employed is given in Table I.

Experiments. In a first series of experiments each group consisted of 15 rats. The experiment was then repeated with 10 animals in each group. The results were almost identical and will therefore be described conjointly.

Since under certain conditions[1] choline chloride appeared to counteract the hypertensive action of anterior pituitary preparation, an additional group of 10 rats was treated exactly as those on Diet I but they received 10 times the amount of choline chloride indicated in Table I.

Results. Table II summarizes our results. The average hypertension given in Table II refers to the average of the hypertensive animals only. It seemed appropriate to us not to include in the calculation the few normotensive rats, in order to obtain the true hypertensive average.

Confirming previous findings of this laboratory[2] it may be seen in Group 2 that the implantation of DCA produced a marked elevation of the blood pressure. This hypertension, which was present in almost all animals, was completely established on the 20th day of the experiment; it persisted for a period of at least 20 days which elapsed between the two blood pressure determinations. The kidney lesions in this group were also very prominent, as judged by the high incidence and severity of the pathologic changes.

We wish to emphasize particularly that a 15% casein diet does not prevent the production of hypertension and nephrosclerosis under these conditions. In fact our results show that at the end of 40 days 100% of the animals in Group 1 were hypertensive, with an average blood pressure of 161 mm Hg and had as marked kidney lesions as in the 30% casein group.

Ten times the usual amount of choline chloride (Group 3) effected no detectable improvement.

Discussion. Our earlier experiments[1] showed that the hypertension produced by anterior pituitary overdosage is prevented by a 15% casein diet; the present observations indicate that DCA-hypertension, on the other hand, cannot be prevented in this manner. According to the working hypothesis prevalent in this laboratory, the anterior pituitary increases the blood pressure by stimulating the adrenal cortex.[4,5] Since it now appears that the DCA-hypertension is obtained even on low protein diets, it is probable that the site of action of dietary proteins is between the pituitary and adrenal cortex: either proteins increase the efficacy of the corticotrophic hormone or they stimulate corticotrophin production. Experiments on hypophysectomized rats are now under way in this Institute to elucidate this last-mentioned point.

Choline chloride had no influence upon the establishment of hypertension by DCA overdosage.

Summary. While the hypertension and nephrosclerosis produced in the rat by anterior pituitary preparations are prevented by low-

[2] Selye, H., and Pentz, E. I., *Canad. M. A. J.*, 1943, 49, 264.

[3] Hay, E. C., and Seguin, P., *Am. J. Physiol.*, 1946, 147, 299.

[4] Selye, H., and Stone, H., *J. Urology*, 1946, 56, 399.

[5] Hall, C. E., Dontigny, P., Beland, E., and Selye, H., *Endocrinology*, 1946, 38, 296.

TABLE II.
Effect of Dietary Protein Concentration upon Hypertension and Nephrosclerosis Produced by Desoxy-corticosterone Acetate Overdosage.
(Averages and standard errors.)

Groups	No. rats at end of exper.	Diets*	Final body wt	Nephrosclerosis		Hypertensive mean pressure		
				Incid. %	Severity %	Avg hypert.†		% hypert. rats. 40 days %
						20 days	40 days	
1	16	15% casein	130 ± 5.2	100	79	154 ± 2.7 (19)	161 ± 3.8 (15)	100
2	13	30% ,,	135 ± 7.0	100	79	160 ± 3.0 (25)	157 ± 5.9 (11)	82
3	8	30% ,, + 10 X choline chl.	144 ± 7.3	100	79	—	163 ± 9.9 (8)	87

* Diets I and II.
† Figures in parentheses refer to number of determinations.

protein diets, the otherwise similar lesions induced by desoxycorticosterone overdosage are largely independent of the dietary protein intake.

16120

Effects of Dithiobiuret on the Central Nervous System.*†

SOL ALTSCHUL. (Introduced by W. S. McCulloch.)

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Astwood et al.[1] reported that 0.002% solution of dithiobiuret, substituted for drinking water, paralyzed and killed rats in 7 days although each took less than .5 mg per diem. Peripheral nerve, myoneural junction and muscle were normal in function. Muscle, peripheral nerve, brain, spinal cord, root and ganglion were microscopically normal. On return to drinking water, the animals promptly recovered, but while paralyzed, strychnine failed to induce convulsions. Effects of di-thiobiuret on cortex and convulsant threshold is reported here.

Method. Dithiobiuret, prepared by the American Cyanamid Co., is sparingly soluble in water and as it goes into solution smells of hydrogen sulfide. Saturated solutions in saline or distilled water were used for local applications, intramuscular and intravenous injection, and 0.001 and 0.002% in tap water orally and intraperitoneally.

Two dogs, 3 cats and a fourth that had received 5 cc saturated solution intramuscularly for 8 days were used to determine effects of dithiobiuret when applied to cortex and injected intramuscularly or intravenously in animals under Dial.‡ Electroencephalograms of the fourth cat and one rat that had received 0.002% dithiobiuret for 9 days were

1 Astwood, E. B., Hughes, A. M., Lubin, M., Vanderlaa, W. P., and Adams, R. D., *Science*, 1945, **102**, 196.

TABLE I.

Rat	Dosage and route, g/100 cc	Toxic change seen in rate	Threshold* prior to drug, volts	Threshold* prior to toxic changes, volts	Threshold* during toxic changes, volts
1	.002 oral	severe	27.9†	50†	70-75†
4	,, ,,	,, (died)	45	50-55	70
5	,, ,,	slight	45	50	55
7	,, ,,	severe (pregnant)	40-45	55	70-75
14	,, ,,	,, (died)	—	—	—
15	,, ,,	,, ,,	—	—	—
16	,, ,,	slight ,,	—	—	—
20	,, ,,	died before results obtained	—	—	—
21	,, ,,	severe	—	—	—
22	,, ,,	,,	—	—	—
8	.002 I.P.	slight (died after inj.)	—	—	—
9	,, ,,	severe	—	—	90
10	,, ,,	,,	—	—.	65
2	.001 oral	not toxic	40	50	—
3	,, ,,	severe	46	45	65
6	,, ,,	slight	45	50	75
17	,, ,,	not toxic	—	—	—
18	,, ,,	,, ,,	—	—	—
19	,, ,,	,, ,,	—	—	—
11	.001 I.P.	severe	—	—	80-85
12	,, ,,	,,	—	—	70
13	,, ,,	,,	—	—	80

* Cortical threshold to electrically induced convulsions.
† A copper electrode was placed into the mouth; in all other rats a brass electrode was used.
I.P. Intraperitoneal.

taken to determine effects of prolonged administration. Normal animals were used as controls. Electrical activity of cortex was recorded by a 6-channel Grass electroencephalograph.

Rats were used to determine effects of dithiobiuret upon cortical threshold to electrically induced convulsions. Convulsions were produced by a Goodwin, condenser discharge, stimulator§ with one electrode placed into the mouth; the other on the vertex of the scalp. Voltage was varied from 25 to 90 volts in 5 volt increments, while frequency of stimulation was 120 per sec. and time duration of the falling phase of each stimulus was 0.5 millisec. Duration of stimulation in all cases was 5 sec. and the interval between attempts to induce convulsions was 20 to 30 min. Cortical threshold to electrically induced convulsions was defined as voltage (other factors remaining constant) required to produce toni-clonic activity lasting more than 5 sec. after stimulation. Sixteen rats received unrestricted amounts of dithiobiuret

§ The Goodwin Stimulator used in this study was obtained through the courtesy of Lab-Tronics, Inc.

orally and 10 rats received daily 10 cc doses of dithiobiuret intraperitoneally. Four rats given 0.002 and 3 rats 0.001% dithiobiuret orally were used to determine cortical threshold before, during, and after administration of the drug. Two rats receiving 0.002 and 3 rats 0.001% dithiobiuret intraperitoneally were used to determine cortical threshold only after toxic changes were seen. (Table I). After toxic changes in the animals were noted, 10% glucose in 10 cc doses were injected intraperitoneally. One dose was given to each of 6 rats and 2 doses were given to each of 2 rats.

Results. Electroencephalographic tracings of 4 cats and 2 dogs did not change after local application of dithiobiuret or after single intravenous and intramuscular injections of saturated solutions of dithiobiuret in doses ranging up to 100 cc. Prolonged administration of dithiobiuret in one cat and one rat failed to produce any significant difference in electrical activity of cortex from that of normal animals.

The following progressive physical changes were noted in rats 5 to 12 days after the

initial dose of dithiobiuret. First was a decreased ability to use their hind limbs, as indicated by a shuffling gait; the animals would lie still most of the time, unless their tails were pinched. Next, they completely lost the ability to use their hind limbs. Finally, the rats could not move at all and died. After toxic changes were first seen, it took from 2 to 7 days for severe changes and death to occur. A slight toxic reaction was defined as the beginning disturbance of gait, while a severe reaction was defined as an inability to use the hind limbs. Of 10 rats receiving 0.002% orally, 7 showed severe reactions, 2 showed slight reactions while one died before changes were noted. Five rats receiving daily intraperitoneal doses of 10 cc of either 0.001 or 0.002% solutions showed the same severe changes, which appeared however, a few days earlier. A sixth rat on 0.002% intraperitoneally died shortly after an injection, but had already displayed slight effects to previous doses. Of 6 rats on 0.001% orally, only 2 showed toxic effects.

With respect to the cortical threshold‖ to electrically induced convulsions, the following results were noted. Prior to administration of dithiobiuret, 6 rats displayed typical toni-clonic muscular contractions of a grand mal type after stimulation at approximately 45 volts. A seventh rat had a cortical threshold of 28 volts. A copper electrode, however, was placed into its mouth while in all others a brass electrode was used. In these 7 rats stimulated after the drug had been given and before toxic changes were seen, a change in cortical threshold varying from a decrease of one volt to an increase in 20 volts was noted (Table I). The average change was a 6-volt rise in threshold. These same animals when showing toxic effects had a rise in the cortical threshold of 10 to 45 volts, with an average rise of 26 volts. The rat showing only 10 volts change died before severe toxic effects were present. In 5 animals, tested only

‖ The term "cortical threshold" is used in the accepted manner, but since a non-blocking amplifier was not available for recording local changes, cortical discharges that were blocked at a lower level, might have occurred prior to observed seizures.

after toxic changes had been observed, the increase in cortical threshold ranged from 20 to 45 volts, with an average increase of 28 volts over the average cortical threshold of animals tested prior to administration of the drug. The form of the convulsions changed when the animals became intoxicated. Non-intoxicated rats responded to stimulation with typical toni-clonic seizures, while seizures in intoxicated rats were decreased in intensity with some animals showing sustained tonic seizures followed by minimal clonic movements. In these animals the voltage was raised until a grand mal type reaction occurred.

Recovery was not hastened by one or two daily intraperitoneal injections of 10 cc of 10% glucose.

Discussion. The effects of dithiobiuret in raising the cortical threshold to electrically induced convulsions is great only when the rats are almost paralyzed.

Since no change in electrical activity of cortex was detected in 4 cats, 2 dogs, and one rat after administration of dithiobiuret, the action can not be based on a disturbance in the electro-chemical system of the cortex responsible for the electroencephalogram.

Astwood *et al.* demonstrated that strychnine failed to induce convulsions in animals paralyzed by dithiobiuret. This, plus findings of ascending spinal paralysis in animals able to respond to painful stimuli, seems to indicate that dithiobiuret produces a functional change in the efferent system of spinal cord.

Summary. The effects of dithiobiuret on electrical activity of cortex were tested upon 4 cats, 2 dogs, and one rat. Normal animals or the same animals were used as controls. Twenty-two rats were used in chronic administration and 12 of these were used to determine cortical threshold to electrically induced convulsions. The significant findings were: (1) a lack of detectable electroencephalographic change in cortical activity of animals tested after immediate or prolonged administration of dithiobiuret, (2) cortical threshold to electrically induced convulsions of rats was raised by an average of approximately 26 volts and (3) the form of con-

vulsion in intoxicated rats was altered.

Conclusion. Dithiobiuret is an effective anti-convulsant in rats only when they are intoxicated. The site of action of dithiobiuret is within the central nervous system; the most probable site is the efferent system of the spinal cord.

16121 P

Specificity of Eczematous Hypersensitivity to P-Aminobenzoic Acid Butyl Ester ("Butesin").

Edward L. Laden and Louis Rubin, (Introduced by S. Rothman.)

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The present study was carried out on a 15-year-old boy who was originally seen for a contact dermatitis resulting from the application of butesin picrate ointment to a burn on the wrist. Routine patch test with the commercial ointment, which contains 1% butesin picrate, yielded a strongly positive reaction with marked reddening, edema and vesiculation.

Experimental and results. Patch tests were carried out in this patient with a series of related compounds in order to establish the range of specificity of this reaction. The materials studied were dissolved in triethanolamine in 2½% concentrations. The patches were removed after 48 hours and the reactions observed for another 48 hours.

Patch tests with a series of homologous p-aminobenzoic acid alkyl esters including methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, and amyl esters yielded positive reactions. Para-aminobenzoic acid and the alcohols which constitute the side chains of the esters yielded negative results. No reaction was obtained with the methyl-ester of meta aminobenzoic acid.

Six commercial anesthetics which are derivatives of p-aminobenzoic acid with a tertiary amine in the side chain were tested with negative results. This series included procaine, larocaine, tutocaine, monocaine, butyn and pontocaine.

Eight local anesthetics, benzoic acid esters with a tertiary amine in the side chain but without an aromatic amino group, were tested.

TABLE I.
Effective Threshold Concentrations of *p*-aminobenzoic Acid Alkyl Esters.

Ester	Effective threshold concentrations
Methyl	1 : 10^2
Ethyl ("benzocaine")	1 : 10^6
Propyl	1 : 10^8
Butyl ("butesin")	1 : 10^6
Amyl	1 : 10^6
Isopropyl	1 : 10^6
Isobutyl	1 : 10^3

These were alypin, metycaine, stovaine, nupercaine, diothane, phenacaine, apothesine and intracaine. All gave negative reactions.

The 7 substances which elicited positive reactions were then tested quantitatively to determine threshold concentrations at which a just perceptible reaction occurs. (Table I)

The table shows that the sensitivity to the methyl ester was relatively low. The sensitivity increased with lengthening of the side chain to reach a maximum with the propyl ester. Further lengthening of the side chains resulted in a decreased sensitivity. The iso compounds caused in both cases considerably less reaction than the isomer n-compounds.

Discussion. There are only a few studies dealing with multiple epidermal sensitivity. In one group of the studied cases, contact dermatitis can be provoked in a rather haphazard fashion by a number of not closely related compounds.[1-4] In the other group, however, the sensitivity has a rather narrow

range within a well defined chemical configuration.[1,4,5,6] Such sensitivity patterns have been perfectly identical in different persons and therefore may be regarded as essential in the mechanism of the sensitization. In the group of procaine sensitive patients, for instance, 3 cases have been reported in which the sensitivity was restricted to those p-aminobenzoic acid derivatives which contain a tertiary amino nitrogen in the side chain.[4,6] The case herein described is similar to 3 cases reported by Schwarzschild[1] in which the sensitivity pattern was restricted to the alkyl esters of p-aminobenzoic acid. Schwarzschild reported in one case that the methyl

ester of p-aminobenzoic acid caused considerably less reaction than the ethyl, propyl and isobutyl esters.

A repeatedly encountered feature of these group sensitivities is that maximum sensitivity is found not with the actually sensitizing compound but with one of the related homologues.[4,6] This was the case in the present observation. The patient was sensitized to the butyl ester but was 100 times more sensitive to the propyl ester with which he apparently never had contact before. The sensitivity patterns studied so far in eczematous reaction can be well explained by Pauling's theory of antibody formation.[7]

Summary. A case is reported with eczematous hypersensitivity restricted to alkyl esters of p-aminobenzoic acid. *No reaction was obtained with substances deviating from this basic structure in the ring or in the side chain.*

[1] Schwarzschild, L., *Arch. Dermat. und Syph.*, 1928, 156, 432.

[2] James, B. M., *J. A. M. A.*, 1931, 97, 440.

[3] Goodman, M. H., *J. Invest. Dermat.*, 1939, 2, 53.

[4] Strauss, M. J., *J. Invest. Dermat.*, 1947, 8, 403.

[5] Rostenberg, A., and Kanof, N. M., *J. Invest. Dermat.*, 1945, 6, 201.

[6] Rothman, S., Orland, F. J., and Flesch, P., *J. Invest. Dermat.*, 1945, 6, 191.

[7] Pauling, L., Campbell, D. H., and Pressman, D., *Physiol. Rev.*, 1943, 23, 203.

16122

Mydriatic Activity of Some New Synthetic Anticholinergic Esters.*

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Numerous publications during recent years have indicated that mydriasis and cycloplegia may be brought about by drugs that are primarily anticholinergic in action. Nyman[1] determined the relative mydriatic activity in man of various alkaloidal and synthetic substances. Scopolamine, 1-hyoscyamine and atropine methyl-nitrate exceeded

the activity of atropine whereas synthetic agents such as trasentine, euphthalmine and di-n-butylcarbaminoylcholine sulfate (dibutoline) are distinctly less active. Fromherz[2] found that the diphenylglycolic acid ester of γ-diethylamino-β,β-dimethylpropanol would produce maximum mydriasis in rabbits when instilled into the conjunctival sac in a concentration of 0.2%. Swan and White[3] have described the mydriatic activity of dibutoline

[1] Nyman, Ebbe, *Acta Physiol. Scand.*, 1942, 3, suppl., 10.

[2] Fromherz, K., *Arch. f. exp. Path. u. Pharmakol.*, 1933, 173, 86.

[3] Swan, K. C., and White, N. G., *J. Pharm. Exp. Therap.*, 1944, 80, 285; *Arch. Ophth.*, 1944, 31, 289; *Am. S. Ophth.*, 1944, 27, 933.

TABLE I.
Spasmolytic Action of Anticholinergic Drugs.

Drug	Structure	Spasmolytic action against acetylcholine induced contractures (Isolated segments of rabbit ileum)
612	β-Diethylaminoethyl di-α-thienylacetate HCl	3 M*
1565	β-Diethylaminoethyl di-α-thienylglycolate HCl	50 M
606	β-Diethylaminoethyl phenyl-α-thienylacetate HCl	4 M
600	β-Diethylaminoethyl phenyl-α-thienylglycolate HCl	60 M
1597	β-Diethylaminoethyl cyclohexyl-α-thienylacetate HCl	17 M
623	β-Diethylaminoethyl cyclohexyl-α-thienylglycolate HCl	100 M
Trasentine	β-Diethylaminoethyl diphenylacetate HCl	1 M
109	β-Diethylaminoethyl diphenylglycolate HCl	33 M
Atropine sulfate		50 M

* Dilution of the drug in parts per million.

and recommend a concentration of 7.5% for clinical use. Ing, Dawes and Wajda[4] determined the mydriatic activity in mice of a large number of new synthetic compounds and found several to be more potent than atropine. However, since these drugs were given intraperitoneally, direct comparison cannot be made with results obtained in other investigations of mydriatic substances wherein the material was applied externally to the eye.

Lands, Nash and Hooper[5] described the pharmacology of a series of anticholinergic esters containing a few drugs with marked mydriatic effects. The synthesis of additional mydriatic drugs has permitted this laboratory to carry out further experiments in an effort to develop synthetic substitutes for atropine and homatropine. A portion of the results obtained is described in this communication.

Results. All drugs were screened for anticholinergic activity by determining the dilution required to abolish acetylcholine contractures in isolated intestinal segments of the rabbit (Magnus), as previously described.[5] Results obtained are shown in Table I. Esters of acetic acid, disubstituted by phenyl, cyclohexyl, or α-thienyl groups, or combinations thereof, were tested for anticholinergic activity. Disubstitution in the acetate portion by unlike groups, such as phenyl/α-thienyl (No. 606) or cyclohexyl/α-thienyl (No.

1597), in the group of compounds tested, gave compounds which were more spasmolytic than either the corresponding diphenyl (trasentine) or di-α-thienyl analogues. The esters of disubstituted hydroxyacetic acid tested were from 6 to 33 times as spasmolytic (anticholinergic) as the nonhydroxy compounds. The most active compound, β-diethylaminoethyl cyclohexyl-α-thienylglycolate HCl (No. 623) exceeded atropine sulfate in spasmolytic potency.

Mydriatic action was determined in albino rabbits selected at random from our colony. The drugs, dissolved in distilled water, were instilled directly into the conjunctival sac; the eyes were held closed for one minute and upon release the excess drug was allowed to drain away. The pupillary response to strong direct illumination (100-watt electric light bulb with reflector held near the eye) was determined before and at intervals after drug instillation. The results obtained are shown in Table II. In general, mydriatic potency parallels spasmolytic potency. The disubstituted acetates, trasentine, No. 612, No. 606 and No. 1597 produce incomplete mydriasis when used at a concentration of 0.5 to 2.0%. The corresponding glycolates are effective mydriatics. No. 623 produces definite mydriasis in rabbits at a concentration of 0.02%. A 0.1% solution produces maximum mydriasis lasting more than an hour with some mydriasis lasting more than 7 but less than 24 hours. With a 1.0% solution there was prolonged mydriasis, corneal anesthesia and moderate irritation of the conjunctival

[4] Ing, H. R., Dawes, G. S., and Wajda, Isabelle, *J. Pharm. Exp. Therap.*, 1945, **85**, 85.

[5] Lands, A. M., Nash, V. L., and Hooper, K. Z., *J. Pharm. Exp. Therap.*, 1946, **86**, 129.

TABLE II.

Mydriatic Action of Anticholinergic Drugs.

$$1.\!-\!\underset{\underset{2.}{1.}}{\overset{\parallel}{\underset{O}{C}}}\!-\!C\!-\!O\!-\!CH_2CH_2N\!\begin{array}{c}C_2H_5\\ \\ C_2H_5\end{array}\cdot HCl$$

Drug	Structure 1.	2.	3.	Conc. %	Mydriatic action	Dur. of recorded mydriasis, min.	Dur. of any mydriasis, hours	Toxicity approx. LD_{50}*
612	α-thienyl	α-thienyl	H	1.00	none		8	500
1565	α-thienyl	α-thienyl	OH	0.10	+, ++	8	1	320
606	phenyl	α-thienyl	H	1.00	+	60		135
600	phenyl	α-thienyl	OH	2.00	++	60-180	>6	
				0.05	+++	30-60	7	
1597	cyclohexyl	α-thienyl	H	0.10	+++	45-60	7	
623	cyclohexyl	α-thienyl	OH	0.50	+	20	1	225
				0.02	+++	60-120	>5	
				0.10	++, +++	60-120	>7, <24	
Trasentine	phenyl	phenyl	H	1.00	+++	120	2-4	194
				2.00	+++	120	3	
109	phenyl	phenyl	OH	0.02	++	120	5	110
				0.05	+++	45-60	5	
Atropine sulfate				0.001	+++	100	7	240
				0.005	++++	120-180	7	

* All drugs were dissolved in distilled water and administered intraperitoneally to mice weighing 14-21 g. The test animals were obtained from our own colony, and both colony and test animals were housed in an air-conditioned laboratory. Deaths occurring during the 72 hours following injection were recorded. At least 30 animals were used for the determination of the LD_{50} of each drug described.

TABLE III.

Salts of β-Diethylaminoethyl Cyclohexyl-α-thienylglycolate.

Salt	M.P., °C	Formula	Analysis Nitrogen calcd.	found	Halogen calcd.	found	Solubility in water 25°C—%	Mydriatic activity 0.02% solution
Hydrobromide	184-185	$C_{18}H_{30}O_3NSBr$	3.33	3.34	19.01	19.03	< 1.0	++, ++, +++
Bitartrate	118-123	$C_{22}H_{35}O_9NS$	2.86	2.77			7.0	++, +++
Nitrate	79-82	$C_{18}H_{30}O_6N_2S$	*				100.0	++, +, +++
Methobromide		$C_{19}H_{32}O_3NSBr$	3.22	3.08	18.39	18.25	>30.0	+++, +, +++
Hydrochloride	174-176†						1.0	+++, +, +++

* Sulfur analysis, calcd. 7.97, found 7.97.
† Blicke and Tsao.[8]

membranes. None of the synthetic drugs described here is more mydriatic than atropine sulfate.

Because of limited solubility of the hydrochloride, other salts were prepared and tested. Results obtained are shown in Table III. The nitrate is completely soluble in water and equals the hydrochloride in its mydriatic potency. A 1.0% aqueous solution of this salt is slightly irritant to the conjunctiva but causes no demonstrable damage to either the cornea or conjunctiva. Solutions at a concentration of 2.0 and 5.0%, made isosmotic to tears by adding glycerine, were instilled into rabbit eyes daily for 5 days. At the end of the test period the eye was bathed with sodium fluorescein and the cornea examined for damage. Irritation was slight with the 2.0% solution and there were only a few fluorescent areas on the corneal surfaces. These were small, being about 1-3 mm in diameter. They disappeared rapidly after treatment was discontinued. The 5.0% solution, similarly applied, caused edema and hyperemia of the conjunctiva and nictitating membrane and by the 5th day of treatment there was a large amount of mucoid discharge and cloudiness in the corneas of the treated eyes. This cloudy area covered half of the corneal surface from the inferior corneal margin upward and was strongly fluorescent when bathed with sodium fluorescein. Recovery was rapid after medication was discontinued and by the 4th day no evidence of corneal damage or irritation could be found.

Gilman *et al.*[6] have described significant local anesthetic effects for β-diethylaminoethyl diphenylglycolate HCl and related structures. The local anesthetic effect of a few of the drugs in our series has been determined in albino rabbits. The drugs were dissolved in distilled water to make a 1.0% solution of the hydrochloride salts and instilled directly into the conjunctival sac of the rabbit eye, as described above for the determination of mydriatic action. The medial surface of the cornea was touched lightly

with a blunted pencil at 2- to 5-minute intervals after drug application. The absence of the wink reflex was taken as an indication of anesthesia. The results obtained were: No. 606—20 min.; No. 600—18 min.; No. 109— 5 to 20 min.; No. 623—43 min. Gilman *et al.*[6] have reported that No. 109 and trasentine, in a 2.0% solution, cause corneal anesthesia for 18 and 21 minutes. No. 606 and 600 appear to have comparable anesthetic effects. No. 623 is distinctly more anesthetic than the above drugs.

Discussion. Lands *et al.*[5] have shown that β-diethylaminoethyl acetate has a cholinergic action on the isolated intestinal segment. Anticholinergic action was observed in those compounds containing an hydroxyl and phenyl, cyclohexyl or α-thienyl groups on the acetate portion of the ester. High spasmolytic potency against acetylcholine induced contractures was associated with high mydriatic potency. Both actions appear to be the result of "blocking" at the cholinergic receptors of the effector cells. Direct comparison of relative effects on these two types of cells is difficult because of the differences in methods used for studying and evaluating the actions described here. The determination of mydriasis was by direct instillation of drugs into the conjunctival sac. The actual concentration of drug in the aqueous humor would be influenced greatly by the rate at which it passed across the corneal barrier and by the rate of absorption from the aqueous humor. On the other hand, the isolated intestinal segments were bathed directly by a known concentration of drug. However, it should be noted that drugs with low potency for the intestinal segment were also poor mydriatics (No. 612, 606 and trasentine).

Blicke and Kaplan[7] have reported results obtained by Dr. John G. Beall with a large group of mydriatic drugs synthesized by them. They report that a 2.0% solution of the γ-dimethylamino-β,β-dimethylpropanol esters of mandelic, β-phenyl-α-hydroxypropionic, β-phenyl-β-hydroxypropionic and β,-β-diphenyl-β-hydroxypropionic acids are with-

6 Gilman, A., Goodman, L., Thomas, J. M., Hahn, G. A., and Prutting, J. M., *J. Pharm. Exp. Therap.*, 1942, 74, 290.

7 Blicke, F. F., and Kaplan, H. M., *J. Am. Chem. Soc.*, 1943, 65, 1967.

out mydriatic action whereas the esters of the corresponding tropic and benzilic acids are strongly mydriatic. The importance of the structure of the acid portion of the molecule is similarly emphasized in our series. The mydriatic and spasmolytic potencies of the β-diethylaminoethyl glycolates described here were much greater than those found for the corresponding acetates. Although two of the drugs described by us (Nos. 600 and 623) have spasmolytic action exceeding that of atropine, none is as strongly mydriatic as atropine. The reasons for this discrepancy are not readily apparent.

Summary. The β-diethylaminoethyl esters of di-α-thienyl-, diphenyl-, phenyl-α-thienyl- and cyclohexyl-α-thienylglycolic acids are anticholinergic drugs with significant mydriatic action. Of these, the cyclohexyl-α-thienylglycolic acid ester (No. 623) is the most mydriatic substance. The nitrate salt of this ester is very soluble in water and causes only small damage to sensitive tissues when used daily for several days at a concentration of 1.0 or 2.0%. It is suggested that this substance may have clinical applications as a mydriatic and cycloplegic agent.

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Enzyme Studies on Human Blood. I. Proteolytic Activity Associated with a Fraction of Plasma.*

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The demonstration of the proteolytic enzyme activity of the blood serum and plasma has attracted the attention of many workers since Dastre[1] originally inferred such an activity in serum. Delezenne and Pozerski[2] reported the proteolytic activity of serum which had been shaken with chloroform. Tagnon[3,4,5] studying this problem more thoroughly found that this enzyme is an euglobulin. Tillett and Garner[6] extracted from cultures of hemolytic streptococci a substance capable of dissolving fibrin clots. Milstone[7] later showed that this material did not act on highly purified fibrinogen and that a plasma euglobulin fraction was necessary for the "fibrinolysis." Christensen[8,10] and Christensen and MacLeod[9] found that the substance isolated from the streptococcic culture acted as an activator ("streptokinase") transforming the inactive plasma enzyme ("plasminogen") into "plasmin." They believe that this "plasmin" differs from trypsin but is identical with the chloroform-activated enzyme. Ferguson et al.[11] employing "fibrinogenolysis" as an assay technic of

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* The protein fractions and the dried whole plasma were obtained through the courtesy of Dr. E. J. Cohn. They were prepared in collaboration between the Department of Physical Chemistry, Harvard Medical School, Boston, Mass., and the Division of Biologic Laboratories of the Massachusetts Department of Public Health from blood collected in collaboration with the American Red Cross.

1 Dastre, 1893, cited by Tagnon,[3] 1942.

2 Delezenne, C., and Pozerski, E., *C. R. Soc. biol.,* 1903, 55, 690.

3 Tagnon, H. J., *Science,* 1942, 95, 334.

4 Tagnon, H. J., Davidson, C. S., and Taylor, F. H. L., *J. Clin. Invest.,* 1942, 21, 525.

5 Kaplan, M. H., Tagnon, H. J., Davidson, C. S., and Taylor, F. H. L., *J. Clin. Invest.,* 1942, 21, 533.

6 Tillett, W. S., and Garner, R. L., *J. Exp. Med.,* 1933, 58, 485.

7 Milstone, H., *J. Immunol.,* 1941, 42, 109.

8 Christensen, L. R., *J. Gen. Physiol.,* 1945, 28, 363.

9 Christensen, L. R., and MacLeod, C. M., *J. Gen. Physiol.,* 1945, 28, 559.

10 Christensen, L. R., *J. Gen. Physiol.,* 1946, 30, 149.

11 Ferguson, J. H., Travis, B. L., and Gerheim, E. B., PROC. SOC. EXP. BIOL. AND MED., 1947, 64, 285.

"tryptic" activity demonstrated such after "streptokinase" activation in Fraction I of human plasma.

Much less conclusive data are available for the demonstration of the active enzyme without previous treatment with "streptokinase," chloroform, or other agents. Schmitz[12-14] isolated an enzyme by adsorption on calcium-fibrin and subsequent fractionation. It produced a slight increase in non-protein nitrogen when added to casein or gelatin. He postulated the existence in plasma of a kinase-trypsin-trypsin inhibitor system. However, recent evidence shows certain dissimilarities between trypsin and the chloroform—or the "streptokinase" activated-enzyme.[9,15] Taylor et al.[16] made observations on the proteolytic activity of plasma protein fractions prepared by low temperature-ethanol separation[17] and without previous chloroform treatment. Qualitatively, the enzyme activity was tested by observing the lytic action of the fractions on a fibrin clot. "Plus" proteolysis is reported in one out of 4 whole plasma samples, 2 out of 6 Fraction I, and in both of Fraction III-2 studied. Edsall[18] states that in Richert's method Fraction III-2 (or III-2,3) is treated with thrombin and the clot which forms adsorbs "plasminogen." This clot lysed spontaneously and was designated Fraction III-3. In 2 or 3 weeks at 0°C a slowly progressive proteolytic activity was demonstrated without the addition of any activator. Glazko[19] also reports the proteolytic activity of Fraction III-3. Christensen[10] observed that fractions of serum containing "plasminogen" will activate spontaneously in the absence of chloroform or "streptokinase" in the refrigerator after several weeks, when the inhibitor content is low.

This report concerns experiments on human whole plasma and its primary protein fractions separated only by low temperature-ethanol procedure and the demonstration of considerable proteolytic activity in Fraction I without previous treatment with activators.

Methods and Material. The dried whole plasma and its fractions were stored in a domestic-type mechanical refrigerator. No special precautions as to temperature, humidity and air exposure were taken during weighing of samples. Unless otherwise specified, a test mixture consisted of equal volumes of 2% aqueous solutions of specified protein fractions and 2% suspension of "C.P." casein in 1/15 M Sorensen phosphate buffer of specified pH, both prepared on the day of incubation. With each test 2 controls were run simultaneously consisting of equal volumes of buffered casein and distilled water and equal volumes of protein solution and buffer. Each test and control mixture was overlayed with C.P. toluene, 1% of the total volume. As additional bacteriological control measure, frequent smears and cultures were taken. The pH was determined with a glass electrode electrometer. Incubation occurred at 37.5°C and the reactions were terminated by the addition of cold 20% trichloracetic acid equal in volume to the reaction mixture. After one hour in the refrigerator, the precipitated proteins were separated by filtration. Either acid soluble total N or "tyrosine" or both were determined in duplicate on one cc aliquots of the filtrate. The micro-Kjeldahl-Nesslerization and the Folin-Ciocalteu[20] procedures, as employed in this communication, have average approximate sensitivities of 0.004 mg N and 0.001 mg tyrosine respectively per 1% on the transmission scale of the Coleman Junior Spectrophotometer. Enzymatic activity is expressed as the mg of acid soluble total N or "tyrosine" liberated per 100 cc of the protein solution obtained by subtracting the amounts found in the enzyme and substrate controls from that found in the

12 Schmitz, A., *Z. physiol. Chem.*, 1936, **244**, 89.

13 Schmitz, A., *Z. physiol. Chem.*, 1937, **250**, 37.

14 Schmitz, A., *Z. physiol. Chem.*, 1938, **255**, 234.

15 Kaplan, M. H., *J. Clin. Invest.*, 1946, **25**, 331.

16 Taylor, F. H. L., Davidson, C. S., Tagnon, H. J., Adams, M. A., MacDonald, A. H., and Minot, G. R., *J. Clin. Invest.*, 1945, **24**, 698.

17 Cohn, E. J., Strong, L. E., Hughes, W. L., Jr., Mulford, D. J., Ashworth, J. N., Melin, M., and Taylor, H. L., *J. Am. Chem. Soc.*, 1946, **68**, 459.

18 Edsall, J. T., *Advances in Protein Chem.*, 1947, **3**, 383.

19 Glazko, A. J., *J. Clin. Invest.*, 1947, **26**, 364.

20 Folin, O., and Ciocalteu, V., *J. Biol. Chem.*, 1927, **73**, 627.

TABLE I.
Control Studies on Protein Solutions and Casein Substrate.

2% protein solution	Principal components*	Acid soluble	
		Total N mg/100 cc†	"Tyrosine" mg/100 cc†
Fraction I	Fibrinogen	5.0	7.6
,, II-III	Gamma and beta globulins	0.8	0.0
,, IV-1	Lipids and alpha globulin	0.4	1.3
,, IV-4	Alpha and beta globulins	0.0	0.0
,, V	Albumin	0.0	0.0
Whole plasma		1.6	1.0
Casein		16.4	14.0

* Information directly from Dr. E. J. Cohn.
† After 48 hrs at 37.5°C, pH 7.1.

TABLE II.
Proteolytic Activity of Fraction I.

Protein solution			Exp.	pH	Incubation, hr	Net acid soluble	
						Total N mg/100 cc	"Tyrosine" mg/100 cc
2%	I	Run 25	A	6.7	48	150.4	
,,	,,	,, ,,	B	6.3	,,	113.6	120.9
,,	,,	,, ,,	,,	6.7	,,	124.6	167.2
,,	,,	,, ,,	,,	7.1	,,	9.4	24.4
,,	,,	,, ,,	,,	7.5	,,	9.6	22.0
,,	,,	,, ,,	,,	7.9	,,	7.6	21.8
0.25%	,,	,, ,,	C	6.7	,,	4.4	2.4
1	,,	,, ,,	,,	,,	,,	10.0	9.8
2	,,	,, ,,	,,	,,	,,	23.0	27.6
,,	,,	,, ,,	D	,,	24		14.2
,,	,,	,, ,,	,,	,,	33		21.2
,,	,,	,, ,,	,,	,,	48		23.5
,,	,,	,, ,,	,,	7.5	24		17.0
,,	,,	,, ,,	,,	,,	33		24.0
,,	,,	,, ,,	,,	,,	48		27.4
,,	I*	,, ,,	,,	6.7	33		—1.3
,,	,,*	,, ,,	,,	,,	48		0.0
,,	I	,, ,,†	E	,,	,,		76.1
,,	,,	,, ,,†	,,	7.5	,,		17.4
,,	,,	,, AVL-20	,,	6.7	,,		18.0
,,	,,	,, ,, ,,	,,	7.5	,,		108.8
,,	,,	,, 186	,,	6.7	,,		92.0
,,	,,	,, ,,	,,	7.5	,,		124.0
,,	,,	,, 8/44	,,	6.7	,,		0.8
,,	,,	,, ,,	,,	7.5	,,		1.0

* Inactivated for 3 min in 80°C water bath.
† Second sample of this preparation received from the Department of Physical Chemistry, Harvard Medical School.

enzyme-substrate mixture.

Results. Table I presents data which emphasize the *necessity* of both enzyme and substrate controls. It is shown that there were present in whole plasma and Fraction I, after 48 hours incubation alone, at pH 7.1, slight but significant amounts of acid soluble total nitrogen and "tyrosine." Similar studies were also made at pH 6.3, 6.7, 7.5, and 7.9 with almost identical results. It was also observed that this amount in Fraction I was constant whether there was spontaneous clot formation and subsequent clot disappearance.[21] There were traces of acid soluble N in Fractions II-III and IV-1 and none in Fractions IV-4 and V. These experiments could be duplicated. However, results with casein substrate alone were variable depend-

[21] Shinowara, G. Y., unpublished observations, 1947.

ing on the particular lot of casein used. The data in Table I represent the highest values obtained. In this laboratory, it has been found that the acid soluble total nitrogen or "tyrosine" of Fraction I alone and of casein alone increases progressively with time of incubation.

Table II contains data on the proteolytic activity of Fraction I upon casein substrate. Experiment A was done 10 days; B, 25 days; C, 36 days; and D, 39 days after receiving the material labeled Run 25 from the Department of Physical Chemistry, Harvard Medical School. Experiment E was done one day after receiving a second shipment composed of material from different runs. With each experiment one or more of the other fractions and whole plasma were run as controls. The summary of results in mg net acid soluble "tyrosine" released from 2% casein by 100 cc of 2% solution of the specified protein at pH 6.3 to 7.9 and 48 hours incubation follows: whole plasma, 5.5 to 13.8; II-III, 6.4 to 12.0; IV-1, 0.0 to 7.8; IV-4, -4.5 to 0.4; and V, -10.2 to 0.0.

The data in experiments A, B, and E, Table II, definitely demonstrate the high degree of proteolytic activity of Fraction I, Run 25, although of a lesser degree, and demonstrate that the activity is apparently dependent upon the period of incubation and the concentration of the enzyme. The one sample of Fraction I, Run 8/44, which did not show enzyme activity differed from the other preparations of this fraction in several respects: It was pure white instead of light amber or gray in appearance; it was more than 90% insoluble in water or saline; and did not clot upon addition of thrombin. Runs 25, AVL, and 186 of Fraction I had clotting times with thrombin between 11 and 14 seconds, whereas Run 8/44, Fraction I, and samples of Fractions II-III, IV-1, IV-4, and V did not clot in 60 seconds.

In Experiment B, Table II, the greatest activity of Fraction I occurred at pH 6.7 and 6.3, but in Experiment D, there was approximately the same amount of proteolysis at pH 6.7 and 7.5. Variable results in pH studies with Fraction I, Runs 25 (second sample received), AVL, and 186 were obtained in Experiment E. Therefore, from the data presented here, it can be stated that apparently the enzyme in Fraction I is active near neutrality. It is also evident from Experiment D that the enzyme activity was completely abolished by heating the 2% solution of Fraction I in an 80° bath for 3 minutes before mixing with the buffered casein substrate.

There was considerably less or no proteolytic activity in the whole plasma and the other fractions tested. No kinases or other activators were added, nor was there a previous treatment with chloroform or fibrin (calcium or thrombin) formation in these and other experiments.

Discussion. The direct demonstration of considerable proteolytic activity of Fraction I was made under analytical conditions intended to give minimal values: These included the use of both enzyme and substrate controls and of cold 20% trichloracetic acid to precipitate the proteins at the end of the incubation period. By careful experiments, Hiller and Van Slyke[22] showed that increasing the concentration of trichloracetic acid increases the precipitation of non-protein nitrogen compounds.

The proteolytic activity of Fraction I can be explained on the basis that the low temperature-ethanol separation resulted in a preparation containing the zymogen and low, if any, concentration of inhibitor. This explanation would be based upon Christensen's[10] hypothesis that the spontaneous activation, without streptokinase, of "plasminogen" occurs when the inhibitor content is low or absent or when the inhibitor has been inactivated by chloroform treatment of serum, resulting in a process which "appears to be autocatalytic in nature." His hypothesis is in agreement with his earlier observation[8] that serum fractions prepared by dilution and acidification to pH 5.3 or by one-third saturation with ammonium sulfate may develop proteolytic activity without kinase or chloroform after a variable period of aging in the cold. The activity was

22 Hiller, A., and Van Slyke, D. D., *J. Biol. Chem.*, 1922, 53, 253.

measured by the gelatin-viscosity method. Christensen and MacLeod[9] concluded that these solutions of inactive serum protease contained little or no inhibitor.

The higher activity of Fraction I at pH range 6.3-6.7 than at 7.1-7.9 in Experiment B, Table II requires some comment. In subsequent Experiments C and D, although significant degree of activity was still present, it had dropped appreciably. A possible explanation is that inadvertently the protein sample was left outside the refrigerator for several hours before Experiment C and this occurred when only a small amount remained in the sample bottle. Christensen[10] reports that in the presence of active enzyme both the "plasminogen" and "plasmin" are destroyed. Nevertheless, the pH studies in experiments B and E may have significance in view of Grob's[23] observation on the shifting of pH optima of leucoprotease and trypsin in the presence of serum from pH 7-8 to pH 6-6.5 as a result of the progressively weakened inhibitor effect of serum upon exposure to pH below 6.5 or above 9.7.

Other preparations exhibiting activity without kinase include Schmitz's[12] calcium-fibrin adsorbed enzyme and the thrombin-fibrin adsorbed Fraction III-3.[18] It appears from the data on these preparations and that reported here that the understanding of the significance of kinase and/or zymogen in the proteolytic system of human plasma *per se* requires further experiments with inhibitor-free kinase, and purer preparations of zymogen, active enzyme, and naturally occurring inhibitors of plasma. Moreover, it is suggested that the liquefaction of a fibrin clot ("fibrinolysis"), the alteration of fibrinogen as measured by clotting time with thrombin ("fibrinogenolysis"), the change in gelatin viscosity, and the increase in acid soluble nitrogen are not representative of an identical enzyme system. In many of the incubation experiments with Fraction I alone "fibrinolysis" and "fibrinogenolysis" were observed while the acid soluble nitrogen did not increase appreciably; *i.e.*, not more than 2% of the total available nitrogen. A comparative study of the various criteria of proteolytic activity will be reported separately. True proteolytic activity (*i.e.*, the cleavage of the peptide bond) of all preparations of Fraction I, which are water and saline soluble, is demonstrated in this report.

Summary. The action of whole dried plasma and its primary fractions separated by low temperature-ethanol procedure on casein was investigated. No kinase was added and there was no previous treatment of the protein preparations with chloroform or any other agents. Proteolytic activity was demonstrated by determinations of acid soluble total nitrogen and "tyrosine."

Human plasma Fraction I contains a high degree of proteolytic activity. The enzyme is inactivated at 80°C and functions at near neutrality.

23 Grob, D., *J. Gen. Physiol.*, 1946, **29**, 219.

16124

Effect of Histolyticus Infection and Toxin on Transplantable Mouse Tumors.*

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The treatment of experimental and human tumors with bacterial infections and bacterial products began with the work of Busch, almost 80 years ago.[1] Since then, the use of bacterial products in the treatment of clinical and experimental cancer has received the attention of many investigators, including Fehleisen,[2] Bruns,[3] Coley,[4] Gratia and Linz,[5] Duran-Reynals,[6] Andervont,[7] and Shear and his co-workers.[8] Brues and Shear[9] reported the treatment of 4 patients with intramuscular injections of a polysaccharide from culture filtrates of *Serratia marcescens* (*B. prodigiosus*), which had previously been shown to produce hemorrhage in and partial or complete destruction of sarcomas in mice.

In previous experiments carried out by two of the present authors, it was demonstrated that in experimental *Cl. welchii* infections in mice,[10] and to an even more marked degree in experimental infections with *Cl. histolyticum*,[11] the systematic administration of the specific antitoxin counteracts the toxemia without preventing the early development of the large local lesions produced by the lytic action of toxins of the gas gangrene group of anaerobes. Accordingly, it seemed reasonable to undertake an investigation designed (a) to destroy tumor tissue by infection with histolyticus spores, to control the resulting toxemia by means of a systemic administration of antitoxin and, finally, to control the infection itself by means of penicillin; (b), to destroy tumor tissue by repeated local injections of small doses of histolyticus toxin; and (c) to test the effect of histolyticus toxin on sarcoma, carcinoma and normal tissues cultivated *in vitro*. Although the results of the experiments that were carried out do not warrant the use of histolyticus infection, or of partially purified histolyticus toxin, in the treatment of human tumors, it is believed that they are of sufficient theoretical interest to justify the present report.

1. *Sarcoma treated with viable histolyticus spores, followed by antitoxin and penicillin. Exp. A.* The first experiment included thirty-three C57 black mice, 18 of which were inoculated with a rapidly growing, transplantable fibrosarcoma (Bar Harbor: L946AII). Of the sarcoma mice, 15 were infected with a spore suspension of *Cl. histolyticum* contained in 0.1 ml of a 5% calcium chloride solution that was injected directly into the developing tumors 8 days after tumor inoculation. Ten of these mice were subsequently treated either with antitoxin (Group 1, 5 animals) or with sodium-penicillin (Group 2, 5 animals) in order to control the infection. Five tumor mice infected with the spore suspension were otherwise untreated (Group 3). Three tumor mice received no treatment whatsoever (Group 4).

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1 Busch, W., *Berl. klin. Woch.*, 1868, 5, 137.

2 Fehleisen, *Deutsch. med. Woch.*, 1882, 8, 553.

3 Bruns, P., *Beitr. klin. Chir.*, 1888, 3, 443.

4 Coley, W. B., *Ann. Surg.*, 1891, 14, 199; *Glasgow Med. J.*, 1936, 126, 49, 128.

5 Gratia, A., and Linz, R., *C. R. Soc. biol.*, 1931, 108, 427.

6 Duran-Reynals, F., PROC. SOC. EXP. BIOL. AND MED., 1933, 31, 341; 1935, 32, 1517.

7 Andervont, H. B., *Am. J. Cancer*, 1936, 27, 77.

8 Shear, M. J., et al., *J. Nat. Cancer Inst.*, 1943, 4, 81, 99, 107, 123; 1944, 4, 461.

9 Brues, A. M., and Shear, M. J., *J. Nat. Cancer Inst.*, 1944, 5, 195.

10 Siebenmann, C. O., and Plummer, H., *J. Pharm. and Exp. Therap.*, 1945, 85, 71.

11 Siebenmann, C. O., and Plummer, H., unpublished experiments.

All treatments were started 24 hours after spore inoculation in order to give the histolyticus infection a chance to develop.

Each mouse treated with antitoxin received subcutaneously 12.5 units daily, for 7 days. The antitoxin consisted of Lederle's polyvalent gas gangrene antitoxin containing 150 units of histolyticus antitoxin per ml. Each mouse treated with penicillin received intramuscularly 250 units twice daily for 2 days, and subsequently, the same dose 4 times daily for 5 days.

The 15 control mice that did not receive tumor inoculations were all infected with spore suspensions. Subsequently, 5 of them were treated with antitoxin (Group 5), 5 were treated with penicillin (Group 6) and 5 were left untreated (Group 7).

In this and subsequent experiments, the tumor material was ground fine enough to be injected through a No. 24 needle. The usual inoculum consisted of 0.5 ml of a 20% suspension in Tyrode's solution made up without bicarbonate.

Results. (Table I). The sarcoma bearers that received no treatment following infection with spore material (Group 3) died early in the experiment, *i.e.*, between the 3rd and 10th day after infection. These early deaths resulted, apparently, from the acute histolyticus infection, for tumor tissue was either in small amount or non-existent. Spore-infected sarcoma bearers that received penicillin (Group 2) developed somewhat larger tumors than those without penicillin. One survivor of this group, which lived until the 16th day, developed a large tumor. But 4 of the 5 spore-infected sarcoma bearers that received antitoxin treatment (Group 1) died from the 15th to the 17th day with little or no tumor tissue evident on macroscopic examination. As in Group 3, these deaths were likely due to histolyticus infection. To the 5th mouse of the group, which survived the 17th day without showing a tumor, penicillin treatment was then given for 6 days in order to eradicate any residual histolyticus infection. The penicillin was given in 20 intramuscular injections of 250 units each. But on the 21st day after spore inoculation, this mouse showed

TABLE I.

Effect of Histolyticus Infection on Sarcoma in Mice (Exp. A).

| | Sarcoma bearing mice | | | | | | | | Normal mice | | | | | |
| | 1 Cl. histolyticum Spores Histolyticus antitoxin (s.c.) | | 2 Cl. histolyticum Spores Penicillin (i.m.) | | 3 Cl. histolyticum Spores None | | 4 None None | | 5 Cl. histolyticum Spores Histolyticus antitoxin (s.c.) | | 6 Cl. histolyticum Spores Penicillin (i.m.) | | 7 Cl. histolyticum Spores None |
Group / Infection / Treatment*	Day of death	Size of sarcoma	Day of death	Size of sarcoma	Day of death	Size of sarcoma	Day of death	Size of sarcoma	Day of death	Infected leg	Day of death	Infected leg	Day of death	Infected leg
Mouse 1	15	trace (L)‡	3	small (L)	3	trace (L)	13	medium	37§	lesion +‖ (healed)	8	lesion ++ (healed)	3	lesion +++
,, 2	15	trace (L)	4	,, (L)	3	,, (L)	28	large	37§	,,	37§	,,	3	,,
,, 3	16	no trace (L)	5	,, (L)	4	no trace (L)	37¶	,,	37§	,,	37§	,,	3	,,
,, 4	17	,, (L)	7	medium	6	,, (L)			37§	,,	37§	,,	3	,,
,, 5	37	large	16	large	10	,, (L)								

* For dosage, see text.
† Number of days after infection.
‡ (L) denotes lysis of tumor.
§ Mouse apparently healthy when sacrificed.
‖ Small lesion +; medium lesion ++; large lesion +++.
¶ Mouse apparently near death when sacrificed.

TABLE II.

Effect of Histolyticus Infection on Sarcoma in Mice (Exp. B).

Group Infection Treatment*		Sarcoma bearing mice								Normal mice				
		1 Cl. histolyticum Spores Histolyticus antitoxin (s.c.)		**2** Cl. histolyticum Spores Penicillin (i.m.)		**3** Cl. histolyticum Spores None		**4** None None		**5** Cl. histolyticum Spores Histolyticus antitoxin (s.c.)		**6** Cl. histolyticum Spores Penicillin (i.m.)		**7** Cl. histolyticum Spores None
Mouse No.		Day of death†	Size of sarcoma	Day of death	Size of sarcoma	Day of death	Size of sarcoma	Day of death	Size of sarcoma	Day of death	Infected leg	Day of death	Infected leg	Day of death
1		3	small (L)‡	3	medium (L)	2	medium (L)	1	small	66§	normal	2	lesion +∥	1
2		4	medium (L)	6	large (L)	2	,, (L)	1	,,	66§	,,	3	,, ++	2
3		4	large	6	,, (L)	2	small (L)	6	large	66§	,,	47	,, +++ (healed)	3
4		5	,,	7	,, (L)	2	medium (L)	7	,,	66§	,,	66§	lesion + (healed)	3
5		6	medium (L)	7	small	3	small (L)	10	,,	66§	,,	66§	normal	3
6		8	,, (L)	8	,,	3	,, (L)	10	,,	66§	,,	66§	,,	4
7		15	large	10	large	3	,, (L)	11	,,	66§	lesion ++ (healed)	66§	lesion + (healed)	5
8		22	medium (L)	11	large	5	,, (L)	11	,,	66§	normal	66§	,,	5
9		25	,, (L)	12	,,	8	large (L)	12	,,	66§	,,	66§	,,	5
10		32	small (L)	12		12	,,	12	,,	66§	lesion ++ (healed)	66§	,,	7

Infected leg (Group 7): lesion +; ,, +++; ,, ++; ,,; ,,; ,,; ,,; ,, +++; ,, ++.

* For dosage, see text.
† Number of days after spore infection.
‡ (L) denotes lysis of tumor.
§ Mouse apparently healthy when sacrificed.
∥ Small lesion +; medium lesion ++; large lesion +++.

a small tumor that grew to full size and resulted in death on the 37th day.

The 5 non-tumor mice that were infected with spore material and treated subsequently with antitoxin (Group 5) showed healed lesions at the time the experiment was terminated on the 37th day. Of the 5 non-tumor mice that were infected with spore material and treated subsequently with penicillin (Group 6), one showed a healed lesion at the time of death, from unknown causes, on the 8th day, whereas the others, also with healed lesions, survived until the termination of the experiment. None of the 5 non-tumor mice infected with spore material without further treatment (Group 7) survived more than 3 days. All showed extensive lysis of the muscles of the infected leg.

Exp. B. As a further means of studying the effect of histolyticus infection on mouse sarcoma, a second experiment was carried out. Except that the groups were larger, this experiment was similar to the previous one in all essential details.

Results (Table II). The sarcoma grew faster in this experiment and killed all the control mice (Group 4) by the 12th day. Of the 10 tumor mice that were infected with histolyticus spores and treated subsequently with antitoxin (Group 1), 4 survived the untreated tumor bearers. In order to suppress any residual histolyticus infection in these 4 survivors, penicillin treatments were begun on the 8th day after infection. The dosage (intramuscular injections of 250 units) ranged from 3,250 units (mouse No. 7) to 6,500 units (mouse No. 10) and depended upon the length of time each mouse survived. All 4 mice finally developed tumors, though in 3 the sarcoma showed definite signs of lysis at the time of death. The mouse that lived longest (mouse No. 10) outlived the last surviving sarcoma-control by 20 days.

II. *Carcinoma treated with viable spores, followed by antitoxin and penicillin.* This experiment, which was carried out simultaneously with Exp. A, and in essentially the same manner, included 33 first generation hybrid mice obtained by mating C57 black females with C3H males. The tumors consisted of transplantable adenocarcinomas (Bar Harbor: E-0771).

Results. Unlike the sarcomas treated in Exps. A and B, the carcinomas showed little evidence of tumor regression following the injection of spore material. With the exception of 2 mice in the group of tumor bearers that received spore material without subsequent treatment, all tumor-bearing mice survived for at least 13 days and developed tumors of considerable size.

III. *Sarcoma treated with toxin.* This experiment included thirty-five C57 black mice, 20 of which were inoculated with the fibrosarcoma (Bar Harbor: L946AII) already mentioned. Of the sarcoma mice, 15 were treated with the toxin of *Cl. histolyticum* administered on the 6th, 8th, 10th and 13th days following tumor inoculation. In 5 of these mice (Group 1), the toxin was introduced directly into the tumor in 4 injections of 1.5 mg each. In 5 mice (Group 2), the same amounts of toxin were injected subcutaneously around the base of the tumor. For the remaining 5 (Group 3), which also received subcutaneous injections around the base of the tumor, the initial injection of toxin consisted of twice the usual amount. Five sarcoma mice (Group 4) received no treatment whatsoever. The 15 control mice that did not receive tumor inoculations were divided into 3 groups of 5 mice each. These groups received the same amount of toxin, respectively, as Groups 1, 2 and 3 of the tumor-bearing mice.

The toxin consisted of a powdered, dry preparation provided by the National Institute of Health (Bethesda, Md.). It was dissolved in saline, distributed in small tubes and kept frozen in a CO_2-ice box until used.

Results. The 5 sarcoma bearers that received no treatment died between the 10th and the 13th day after tumor inoculation; and those that died after the 10th day showed large tumors. There were no deaths among the 15 non-tumor control mice that received toxin injections, although 10 of them developed lesions that eventually healed. Of the 5 tumor-bearing mice that were injected with toxin introduced directly into the devel-

oping tumors (Group 1), 2 died on the 7th day following tumor inoculation (1 day after the beginning of treatment) and the others survived for 13, 14 and 29 days, respectively. The 5 tumor bearers that were injected with toxin introduced about the base of the tumor (Group 2) died between the 14th and 31st days following tumor inoculation. Of the 5 tumor bearers that were injected with toxin introduced subcutaneously about the base of the tumor, but with a larger initial dose (Group 3), one died on the 7th day after tumor inoculation, and 4 survived from 32 to 36 days. Two mice of Group 2 and two mice of Group 3 showed tumors that were only about half the size of those observed in the untreated control mice, whereas the other mice of these groups developed large tumors. In Group 1, in which the toxin was injected directly into the tumors, the 3 mice that survived the treatment for 7 days or more showed an even greater reduction in tumor size.

IV. *Carcinoma treated with toxin.* Although a large series of experiments were made in an effort to test the effect of histolyticus toxin on carcinoma, only one will be reported in detail. This experiment included 16 first generation hybrid mice obtained by mating C57 black females with C3H males. Twelve of these mice were inoculated with a slow-growing, transplantable adenocarcinoma that had arisen spontaneously in a female of the C3H stock. Of the 12 tumor-bearing mice, 4 were treated with a total of 2.5 mg of histolyticus toxin that was introduced directly into the tumor in 8 injections over a period of 20 days, 4 were treated with the same amount of toxin that was introduced intramuscularly but into the leg opposite the one bearing the tumor, and 4 were left untreated. Four control mice without tumors were treated with a total of 4.0 mg of toxin given in 8 injections over 20 days. This was an amount of toxin in excess of that used in treating the tumor-bearers.

Results. On the 7th day following the beginning of toxin treatment (30 days after tumor inoculation), there were 3 deaths in the group that had been injected with toxin

FIG. 1.
Hybrid mice (C57 black x C3H) inoculated with slow-growing, transplantable adenocarcinoma and subsequently treated with 2.5 mg of histolyticus toxin in 8 injections over period of 20 days. (A) Toxin injected directly into tumor, which weighed 0.25 g when photographed; (B) Control, untreated; (C) Toxin injected intramuscularly into leg opposite the one bearing the tumor.

introduced directly into the tumors. At the time of death the tumors were bean-sized, 2 with slight lesions and one with a healed lesion. The remaining mouse of the group carried a small tumor that weighed 0.25 g at the termination of the experiment (Fig. 1A) 44 days after the tumor had been initiated and 21 days following the beginning of toxin treatment. The 4 tumor-bearing mice that had not been treated with toxin developed large tumors that averaged 7.25 g in weight at the termination of the experiment (Fig. 1B). The 4 tumor bearers that had been treated with toxin injected intramuscularly into the opposite leg also developed large tumors that averaged 6.56 g at the termination of the experiment (Fig. 1C).

V. *In vitro studies.* An effort was made to test the effect of histolyticus toxin on sarcoma, carcinoma and normal tissues cultivated *in vitro*, and also, by the same means, to demonstrate the protective action of antitoxin. Kidney was chosen as the normal tissue because it yields, in tissue culture, an abundant growth of both epithelium and connective tissue. The majority of the experiments were carried out in large Carrel flasks (5 cm in diameter) in which 3 or more fragments of sarcoma (Bar Harbor, L946-AII), of carcinoma (Bar Harbor, E-0771), and of young, adult mouse kidney were em-

bedded in 2.0 cc of medium consisting of 0.5 cc of chicken plasma, 0.2 cc of toxin, 0.4 cc of antitoxin and 0.9 cc of a mixture comprised of 40% horse serum (heated ½ hr. at 56°C), 40% Earle's solution, 20% chick embryo tissue juice and sufficient phenol red to make a final concentration of 0.005%. In the control cultures, the toxin and antitoxin were replaced by Earle's solution. The cultures were adjusted with gas mixtures to pH 7.2 and incubated without further treatment for 6 days.

When the medium contained both toxin and antitoxin, the toxin used in the various cultures of a typical series ranged in amount from 0.1 mg to 2.0 mg per cc. When the toxin was used alone, the amounts ranged from 0.01 mg to 1.0 mg per cc. Antitoxin, when present, was in the amount of 0.2 units per cc.

Results. In general, it may be said that the carcinoma was more resistant to the action of the toxin than sarcoma, just as· normal epithelium seemed to be more resistant than stroma cells or fibroblasts. And normal and cancerous epithelium were equally resistant to concentrations of toxin that produced severe damage to the sarcoma cells.

In effective concentrations of toxin, the fibroblast-like sarcoma cells in the zone of outgrowth (or outward migration) rounded up, their nuclei and cytoplasm underwent a pronounced shrinkage, and they proceeded to disintegrate. But the cells of the explant were also affected. They too rounded up, underwent shrinkage and became dissociated into separate entities quite devoid of visible intercellular connections.

Discussion. With reference to the action of histolyticus infection on transplantable tumors, two possible effects were foreseen. The local infection of the tumor tissue might take the course of an acute anaerobic infection, which leads to toxemia and results in the early death of the animal host. Or, the local infection might, apart from any injurious effect on the animal, cause lysis of the tumor tissue and thereby prolong the life of the animal. And because earlier observations on histolyticus infections had shown that antitoxin

counteracts the systemic effect of the toxin without eliminating, necessarily, its lytic action at the site of infection,[11] it seemed reasonable to study the effect of the antitoxin-treated infection on developing mouse tumors.

Both experiments in which sarcoma was treated with spores showed that the uncontrolled infection led more rapidly to the death of the animal than when the tumors were left untreated. The same held true for spore infected tumors treated with penicillin. The effect of the antitoxin treatment, however, was to prevent or postpone death from toxemia; and, as had been expected, it left enough of the local infection to cause a more or less marked lysis of the tumor tissue, thereby prolonging the life of some of the tumor bearers considerably beyond the life span of the non-infected tumor controls.

In order to determine whether, in such survivors, the histolyticus infection had completely destroyed all tumor tissue, the infection was finally eliminated by means of prolonged penicillin therapy. But none of the antitoxin treated mice, subsequently given penicillin for this purpose, survived the test; sooner or later they all developed sarcomas from which they died.

It has been noted that in the experiment in which carcinoma was treated with spore material followed by antitoxin and, finally, penicillin, these tumors, unlike the sarcomas, showed little or no regression. But it was impossible to determine, from the limited observations that were made, whether the greater resistance of the carcinoma was due to a greater resistance of the constituent cells or to the peculiar structure of the tumor itself. From the tissue culture experiments that were made with toxin, it would seem that the carcinoma is actually more resistant than the sarcoma, and that the greater susceptibility of the sarcoma may possibly be due to a greater looseness in the architecture of this type of tumor. In the cultures, the sheets of epithelial cells that grew out from the fragments of carcinoma survived concentrations of toxin that were definitely injurious to the sarcoma cells. But normal epithelium, growing side by side with the malignant epithelium,

was likewise resistant to concentrations of toxin that killed the sarcoma cells. The spindle-like sarcoma cells grow in an open meshwork, whereas carcinoma and normal epithelium grow in a compact mosaic in which the individual cells are far less vulnerable to outside influences.

In so far as the animal experiments with toxin are concerned, there is little that need be added to the observations already made. Just as the antitoxin-controlled histolyticus infection has, under certain conditions, a retarding influence on the development of the tumors, so also are the tumors susceptible to carefully regulated injections of toxin, the amount of which must be sufficient to produce lysis of the tumor but not enough to cause the death of the host. But regardless of how completely a tumor may seem to have regressed, it is almost certain to recur once the treatments have ceased. To be sure, the absorption of toxin does seem to be more rapid in tumor tissue than in normal tissue. Aside from the direct effect of the toxin on the tumor cells themselves, there is also damage to the young, actively proliferating vascular system of the tumor, and this results in hemorrhage and necrosis and the general destruction of the tumor mass. And as further evidence that the effect of histolyticus toxin on tumor cells is not a specific one, systemic injections of toxin at concentrations below the level that is injurious to the host have no effect on the tumors, just as toxin injected around the base of a developing tumor produces less lysis of the tumor than toxin injected directly into the tumor mass.

The main difficulty in attempting to evaluate the effect of histolyticus toxin on experimental tumors, in the present experiments, is the fact that the minimal effective dose approximated, very closely, the lethal dose.

For further study, a more highly purified preparation of toxin would be required, and an attempt should be made to isolate, from the crude toxin, a factor that would be both highly histolytic and of low systemic toxicity.

Summary. When transplantable mouse sarcomas were infected experimentally with *Cl. histolyticum*, and the infection controlled by systemic injections of histolyticus antitoxin, the life span of some of the animals was prolonged for as long as 20 days beyond that of the non-infected tumor bearers. But in no instance did the infection completely destroy the tumor tissue. All surviving mice, treated eventually with penicillin in order to eradicate residual infection, developed large sarcomas. Under similar conditions, no such temporary regression was observed for transplantable mouse carcinomas.

When a study was made of the effect of histolyticus toxin on transplantable mouse tumors, it was found that systemic (intramuscular) injections of toxin were without effect, whereas repeated local injections of toxin, given either directly into the tumor mass or subcutaneously around the base of the tumor, resulted in marked regression of tumor tissue. But for the toxin preparations used, the effective dose approximated, very closely, the lethal dose, particularly when the toxin was injected directly into the tumor mass. In no instance was there a permanent regression of the tumor.

In tissue culture, carcinoma cells were more resistant to the action of histolyticus toxin than sarcoma cells, just as normal epithelium seemed to be more resistant that stroma cells or fibroblasts. But normal and cancerous epithelium were equally resistant to concentrations of toxin that produced severe damage to sarcoma cells.

16125

Comparison of Two Types of Permanent External Bile-fiistula Dogs for Studying Liver Function.

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Studies of hepatic physiology involving the use of dogs with the usual types of permanent external bile fistula are open to criticism because of the great difficulty of maintaining normal liver function in such animals except for short intervals. This is emphasized by the recent report by Drill[1] and is borne out by our experience during the course of previous studies of the excretion of bromsulfalein in bile.[2,3,4] Only 6 of 22 bile-fistula dogs prepared for this purpose proved to be satisfactory for the establishment of criteria for normal excretion of the dye. The purpose of the present communication is to indicate the usefulness of another type of fistula dog for such studies.

Materials and Methods. The two types of permanent biliary fistula employed were the standard type (Rous-McMaster[5]) as modified by Beerman et al.[6] and the type in which a Thomas intestinal fistula is placed opposite the papilla of Vater.[7,8] In the former, following cholecystectomy, a rubber tube was placed in the cystic duct with the common duct tied, or in the common duct with the cystic duct tied, and brought out through a stab wound in the abdominal wall. Bile drained into an attached rubber bag and was reintroduced into the duodenum through a small indwelling duodenal catheter. These dogs recovered slowly after operation, required a special diet and daily care. Because they were prone to develop biliary obstruction they usually survived for only a few weeks or months. As a rule the obstructive phenomena were not pronounced and were unrecognized until the excretion of bromsulfalein in the bile was tested or jaundice developed.

The Thomas intestinal fistula was prepared following cholecystectomy by placing a permanent metal cannula in the duodenum opposite the papilla of Vater. With the cannula open a special glass catheter[9,10] (modified by Thomas from those originally devised by Scott), was introduced when desired into the ampulla of Vater for the drainage of bile. Upon termination of the experimental period the catheter was withdrawn and the cannula closed, which prevented further loss of intestinal contents. These dogs recovered promptly from the operation, did not require special diet or care and survived for long periods, some for over 2 years, without developing biliary obstruction.

Studies were made on 7 dogs with standard type fistulas (11 tests) and on 3 dogs with Thomas type fistulas (19 tests). All of these dogs were given intravenous injections of 2 mg bromsulfalein per kilo of body weight. Four additional Thomas-type fistula dogs were studied by comparing the excretion of bromsulfalein injected in quantities of 2 mg and 5 mg per kilo of body weight.

Bile was collected in 15-minute periods for 2 hours subsequently. Determinations were

1 Drill, V. A., Annegers, J. A., Snapp, E. F., and Ivy, A. C., *J. Clin. Invest.*, 1945, **24**, 97.

2 Cantarow, A., and Wirts, C. W., Jr., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 252.

3 Wirts, C. W., Jr., and Cantarow, A., *Am. J. Dig. Dis.*, 1942, **9**, 101.

4 Cantarow, A., and Wirts, C. W., Jr., *Am. J. Dig. Dis.*, 1943, **10**, 261.

5 Rous, P., and McMaster, P. D., *J. Exp. Med.*, 1923, **37**, 11.

6 Berman, A. L., Snapp, E., Ivy, A. C., Atkinson, A. J., and Hough, V. S., *Am. J. Dig. Dis.*, 1940, **7**, 333.

7 Thomas, J. E., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 260.

8 Snape, W. J., and Thomas, J. E., *Fed. Proc.*, 1945, **4**, 66.

9 Hart, W. M., and Thomas, J. E., *Gastroenterol.*, 1945, **4**, 409.

10 Scott, V. B., Collingnon, U. J., Bugel, H. J., and Johnson, G. C., *Am. J. Physiol.*, 1941, **134**, 208.

TABLE I.

Excretion of Bromsulfalein in the Two Types of Fistula-dogs Using 2 mg of Dye per Kilo of Body Weight.

	Standard fistula dogs		Thomas-type fistula dogs
	Unselected	Selected	
Range of conc. (mg dye per 100 cc bile)	1 to 476	1 to 532	6 to 540
Avg % excreted			
1 hr	5.9	43.6	45.7
2 hr	18.1	65.0	75.0
Vol. of bile (cc) per hour	7.4	—	7.7

Excretion of Bromsulfalein in Thomas-type Fistula Dogs Using 2 mg and 5 mg of Dye per Kilo of Body Weight.

	5 mg dosage	2 mg dosage
Range of conc. (mg dye per 100 cc bile)	5 to 1430	6 to 540
% excreted		
1 hr	51.2	45.7
2 hr	64.1	75.0
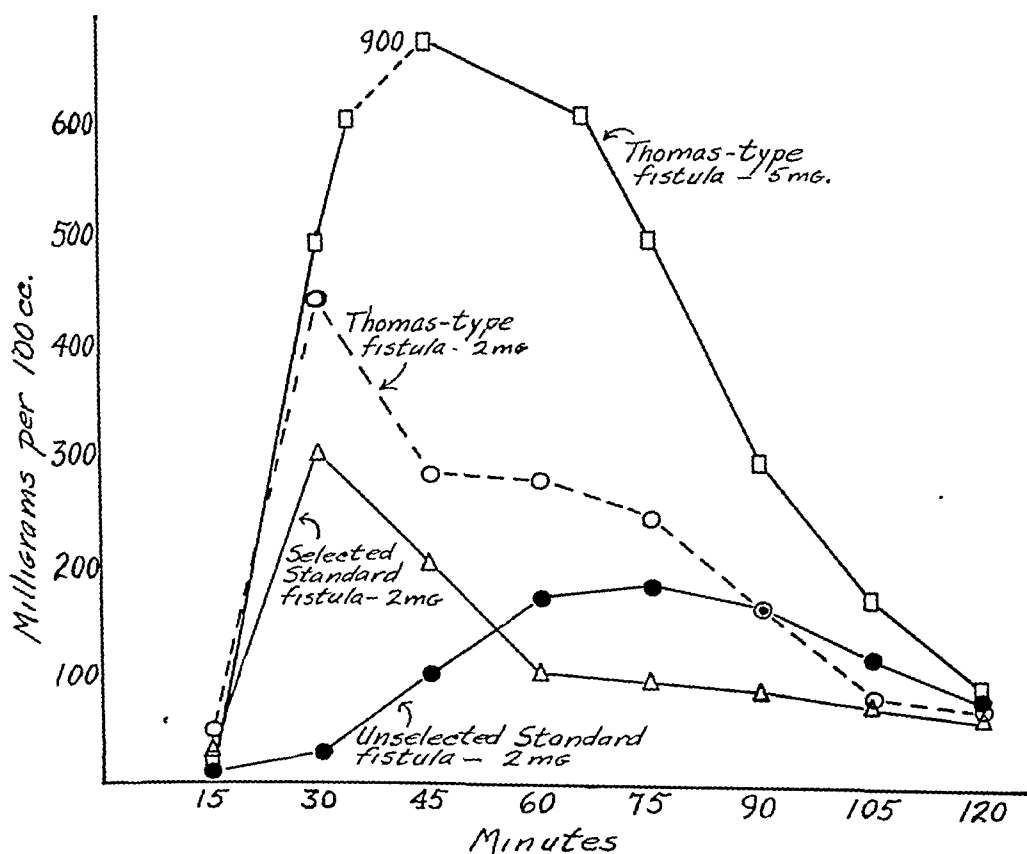
FIG. 1.

Curves of concentration of bromsulfalein in the bile of bile fistula dogs, after injection of 2 mg and 5 mg per kilo of body weight.

made of the concentration and amount of bromsulfalein in each sample. The following criteria were used as a basis of comparison of the two groups of animals: (1) completeness of removal of dye from the blood; (2) rate of entrance of dye into the bile; (3) time of attainment of maximum concentration of dye in the bile; (4) curve of excretion of dye in the bile; (5) range of concentration of dye in the bile and (6) total dye excretion in the bile within one- and 2-hour periods following injection.

Prior to initiating a series of liver function studies the two types of permanent biliary fistula dogs were compared for suitability by observing their prompt convalescence from operation, maintenance of good general physical condition and liver function. The liver function was tested by estimating the quantity and rate of excretion in the bile of intravenously injected bromsulfalein and, in some animals, estimating the quantity of dye retained in the blood according to a method previously described.[2]

Results. The pertinent data are summarized in the table and figure. No abnormal retention of dye in the blood was observed in the Thomas-type fistula dogs; abnormal retention occurred in 5 of 6 standard fistula dogs. Similary, the unselected dogs with the Thomas-type fistula were superior to unselected dogs with the standard type fistula when judged by the other criteria enumerated above. Dogs in the latter group selected on the basis of satisfactory bromsulfalein excretion approached the former in this regard.

Summary and Conclusions. Evidence has been presented to indicate that dogs with the Thomas-type bile fistula are much more satisfactory than those with the Rous-McMaster-type fistula for studies of liver function, particularly those involving collection and examination of bile. Data are presented regarding the normal biliary excretion of bromsulfalein following intravenous injection of 2 mg and 5 mg of the dye per kilogram of body weight.

16126 P

Urticarial Hypersensitivity to Sunlight.

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Urticarial hypersensitivity to sunlight or solar urticaria is a well recognized disease entity.[1] The reaction is analogous to the urticarial hypersensitivity reactions to foods, drugs, and such physical agents as mechanical, cold or heat stimuli. These reactions have all the characteristics of Thomas Lewis' "triple response".[2] It is generally assumed that in this type of hypersensitivity the antigen-antibody reaction leads to wheal formation via the liberation of histamine. Solar urticaria has been regarded as one form of the "physical allergies" in which an antigen or allergen is produced by action of a physical agent. Recently two types of solar urticaria have been described.[3,4] In one type the sensitivity spectrum is between λ 4000-5000 Å and in the other type it is below λ 3700 Å.

Experimental. In this clinic 2 patients were studied who responded to sunlight exposure with severe urticarial reaction. In both cases

1 Blum, H. F., *Photodynamic Action and Diseases Caused by Light*, New York, Reinhold Publ. Corp., 1941.

2 Lewis, T., *Blood Vessels of the Human Skin and Their Response*, London, Shaw and Sons, 1927.

3 Abramson, H. A., PROC. SOC. EXP. BIOL. AND MED., 1940, 43, 410.

4 Blum, H. F., Baer, R. L., and Sulzberger, M. B., J. Invest. Derm., 1946, 7, 99.

it was possible to establish the spectral sensitivity: (1) by radiating the skin of the patients through a set of Corning glass filters and (2) by use of a monochromator which made it possible to project single lines of the mercury arc spectrum on the skin. In both cases the range of sensitivity was found to be between 2967 and 3341 Å with a maximum sensitivity at 3131 Å. Thus these 2 patients belong to the type $< \lambda$ 3700 Å.[4]

The urticarial sensitivity could be passively transferred. By injecting the patients' serum into the skin of normal individuals and irradiating the injection site with the active wavelengths, a "triple response" could be elicited. The transfer site began to manifest sensitivity within 30 minutes after injection and remained sensitive for several days although the sensitivity decreased with time. Normal control sera never caused such reactions.

The incidence of successful passive transfers was much higher with the serum of the more sensitive patient than with the serum of the less sensitive one. When large surfaces of the body were exposed to ultraviolet light prior to withdrawal of blood for passive transfer experiments, the incidence of positive transfers was greatly increased.

Studies of the properties of the transferable sensitizing agent in the serum revealed that it decreased in potency with storage at icebox temperature. After 8 days cold storage of otherwise potent serum, samples gave only weak or questionable passive transfer tests. The sensitizing agent proved to be heat labile and was inactivated at 56°C in ½ hour. Also, the agent was inactivated by irradiation with ultraviolet light. Evidence that the photosensitizing agent is a large molecule, probably of colloid nature, is suggestive since it failed to dialyze through a semi-permeable membrane.

Early morning gastric analysis following radiation of large areas of the patients' skin showed a rise in total acidity in both patients.

A number of substances with absorption spectra similar to the sensitivity spectrum of the patients were incorporated into ointments and tested for protective action against the active rays. Some protection was obtained by using an ointment containing 30% G-Salt (sodium salt of 2 - naphthol - 6, 8 - disulfonic acid).

Greater protection was achieved by the oral administration of antihistaminic drugs, benadryl and pyribenzamine. By virtue of the protective action of these "antihistamines" it was possible to subject the patients to gradually increasing generalized ultraviolet light exposures.[5] By this treatment the skin acquired such tolerance that the administration of "antihistamines" could be discontinued. This gradual increase in tolerance to the active wavelengths was purely a local effect since no protection was achieved on skin areas which were kept covered during the course of ultraviolet therapy. The protection may have resulted from pigmentation and/or the thickening of the horny layer of the skin. Both of these physiologic reactions to sunshine hinder the penetration of the active wavelengths.

Discussion. The narrow and highly specific range of the active wavelengths in this condition together with the passive transferability of the sensitivity to normal individuals with the patients' serum strongly supports the theory of Sulzberger and Baer.[6] According to these authors, the absorption of the specific wavelengths causes the formation of a photochemical product in everybody's skin; however, patients with solar urticaria develop a sensitivity by forming antibodies against this normal metabolite.

Summary. In 2 cases of solar urticaria the maximum sensitivity was found to be at 3131 Å. Studies of the photosensitizing agent in the patients' serum support the conception that this condition is due to sensitization to a physiological radiation product in the skin.

5 Rubin, L., Beal, P. L., and Rothman, S., *J. Invest. Derm.*, 1947, 8, 189.

6 Sulzberger, M. B., and Baer, R. L., *J. Invest. Derm.*, 1945, 6, 345.

16127

Studies on Uterine Metabolism. I. Adenosinetriphosphatase Activity of Smooth Muscle.

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The activity of the adenosinetriphosphatase (ATP-ase) enzyme system has been measured in many tissues other than skeletal muscle. DuBois and Potter[1] have indicated that an unidentified smooth muscle yielded, in their tests, about one-third of the ATP-ase activity of striated muscle. We have investigated rather extensively the ATP-ase system in the smooth muscles of the rat, in particular the uterus. ATP was prepared as the dicalcium salt according to Kerr.[2]

The reaction systems were incubated at 37°C for 15 minutes in a final volume of 3 ml, and contained one ml of a tissue homogenate[1] in 0.1 M veronal-HCl buffer of pH 8.4; one ml of a 10^{-3} molar solution of ATP and one ml of veronal-HCl buffer. Magnesium ions were present in the system in a final concentration of 10^{-4} molar.[3] Blanks were run with ATP alone, and also with tissue in the absence of ATP. Addition of other substances was compensated for in volume by a corresponding reduction in the volume of buffer. The liberated phosphate was determined by the method of Fiske and Subba-Row.[4]

Table I expresses the results found for ATP-ase activity of smooth and skeletal muscles of adult Sprague-Dawley rats. These were all used in the metestrus stage as determined by the daily vaginal smear technique. Varying amounts of tissue were used to insure a linear relationship between tissue weight and enzyme concentration.

The larger variations in the smooth muscle

determinations were thought to be due to the water content fluctuations which these tissues undergo, depending upon their immediate physiological states. However, calculation of the data on the basis of dry weights yielded similarly extensive variations. It must, therefore, be assumed that these changes arise through some other mechanism.

It is significant, however, that the smooth muscles in these systems exhibit a uniformly higher activity than striated muscle. In later experiments striated muscle was often tested at the same time as smooth muscle from the same animal; the above differences in enzymatic activity were always observed to hold true.

The contractile system of smooth muscle remains considerably more obscure than that of skeletal muscle. If the ATP-ase system of smooth muscle is to follow the same myosin-adenosinetriphosphate changes postulated for striated muscle[5,6] the sensitivities of these systems to chemical mediators such as acetylcholine and epinephrine should be similar. Ziff[7] has reported that acetylcholine, epinephrine and eserine are without effect on the hydrolysis of ATP by myosin, which we assume he obtained from skeletal muscle. We are able to corroborate his findings for striated muscle and extend them to uterine muscle. The concentration of the various mediators used is of the same order of magnitude as that employed to elicit physiological responses *in vitro*. The data are given in Table II.

These variations are not considered significant in the light of changes in enzymatic

[1] DuBois, K. P., and Potter, V. R., *J. Biol. Chem.*, 1943, **150**, 185.

[2] Kerr, S. E., *J. Biol. Chem.*, 1941, **139**, 121.

[3] Singher, H. O., and Meister, A., *J. Biol. Chem.*, 1945, **159**, 491.

[4] Fiske, C. H., and SubbaRow, Y., *J. Biol. Chem.*, 1925, **66**, 375.

[5] Engelhardt, V. A., *Advances in Enzymology*, 1946, **6**, 147.

[6] Szent-Györgyi, A. von, *Bull. soc. chim. biol.*, 1943, **25**, 242.

[7] Ziff, M., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 249.

TABLE I.
ATP-ase Activity of Smooth and Skeletal Muscle.
Values expressed as μg P liberated in 15 min.

Tissue homogenate	No. of trials	Per mg fresh tissue		Per mg dry tissue	
		Avg	Range	Avg	Range
Skeletal muscle (Sartorius)	9	8.1	(5.8-13.2)	33.7	(25.0- 56.0)
Intestine (1st loop of duodenum)	6	18.0	(14.0-24.2)	61.8	(46.9- 78.0)
Bladder	4	20.9	(16.6-23.2)	81.8	(73.0-103.0)
Uterus	6	20.8	(16.8-24.2)	89.0	(62.6-110.0)

TABLE II.
Influence of Chemical Mediators on ATP-ase Activity of the Uterus.

Substance	Final conc.	Estrus stage	Avg % change in ATP-ase activity of uterus
Acetylcholine	7×10^{-9} M	Metestrus	+1.5
		Diestrus	—4.4
Epinephrine	1×10^{-7} M	Metestrus	+6.3
		Diestrus	+2.4
Eserine	4×10^{-9} M	Diestrus	+1.4

activity of tissues to which the above substances have not been added. As far as these *in vitro* tests go it may be concluded, therefore, that in skeletal and smooth muscle, these mediators do not effect the ATP-ase system *per se*. The activation of ATP-ase by acetylcholine, described by DuBois and Potter[8] was carried out in a system in which the calcium concentration was a factor which is not the case in these experiments.

Consistent changes in the activity of ATP-ase in smooth or skeletal muscle could not be demonstrated with histamine, pitocin or ammonium chloride. Ethyl alcohol (0.7 M) inhibited the system slightly but this is probably due to its protein denaturant properties. Engelhardt,[5] Ziff,[7] and others[9] have indicated that the inhibitory effect of fluoride is only as great as its removal of calcium ions from the medium. In the magnesium activated system we have observed inconsistent effects of fluoride on striated muscle. Uterine muscle ATP-ase, however, is usually inhibited, as shown in Table III.

The ATP-ase activity of the uterus has led us to investigate the effect of the reproductive cycle on such activity. Because of the water shifts it seemed best to calculate

TABLE III.
Effect of 3.3×10^{-3} M Fluoride on ATP-ase System of Smooth Muscle.

Stage of cycle	% change of ATP-ase activity
Estrus	—25.8; —37.8
Metestrus	—15.5
Diestrus	—17.4; —40.7
Proestrus	—18.6

activity on the basis of dry weight of the tissue used. Later experiments have also included per cent nitrogen as a factor. The results from preliminary experiments do not lend themselves to an interpretation of variation with the cyclical changes, as indicated in Table IV.

Representative steroid hormones were added to the incubate in concentrations of between 10^{-4} to 10^{-6} molar. Additions were made in a small amount of alcohol, and alcohol controls run simultaneously. Estrone, estradiol and progesterone were without appreciable effect on the uterus in the metestrus stage; the latter two also had negligible effect during diestrus. In a small number of cases testosterone seemed to inhibit the ATP-ase activity of skeletal muscle; its effect on uterine muscle appeared to be much smaller.

Summary. The ATP-ase activity of smooth muscle is found to be considerably higher than that of striated muscle. It is unaffected by dilute concentrations of chemical mediators. Fluoride apparently has an inhibitory

8 DuBois, K. P., and Potter, V. R., *J. Biol. Chem.*, 1943, **148**, 451.

9 Polis, B. D., and Meyerhof, O., *J. Biol. Chem.*, 1947, **169**, 389.

TABLE IV.

ATP-ase of the Uterus Through the Estrus Cycle.

µg Inorganic P Liberated in 15 min per mg Dry Tissue.

		Skeletal muscle		Uterus	
Stage	No. of trials	Avg	Range	Avg	Range
Estrus	2	35.5	(35-36)	125.0	(120-130)
Metestrus	9	33.7	(25-56)	93.9	(62-122)
Diestrus	3	50.0	(32-52)	107.3	(63-143)
Proestrus	2	35.5	(17-54)	106.0	(88-140)

effect. Variation in the ATP-ase activity of the uterus could not be correlated with cyclical estrus change. With the possible excep- tion of testosterone, hormones did not appear to affect the *in vitro* systems.

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Production of Essential Fatty Acid Deficiency Symptoms in the Mature Rat.*

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In the course of studies on the effect of vitamin imbalance under various conditions, mature rats were maintained on restricted caloric intake of fat free diets until they lost about 50% of their original weight. In the period of recovery, during which time they were fed the same diets *ad libitum*, various symptoms including those typical of the Burr and Burr syndrome[1] were observed.

Since the original report by Burr and Burr,[2] several workers[3,4,5] have observed the typical skin symptoms of the essential fatty acid deficiency, but in all cases young animals were used. So far as we know, in no case was this deficiency syndrome reported to have been produced in adult rats.

Experimental and results. Mature male rats of the Sprague-Dawley strain averaging about 225 g have been used in these experi- ments. The rats were placed in individual metal cages with raised screen bottoms at the beginning of the depletion period on the fat free diets. They were weighed and examined weekly or in later experiments twice weekly.

In the first experiment 4 groups of 4 rats each were placed on the 4 diets given in Table I. In addition they all received one drop of haliver oil weekly.

For a period of 2 months each rat received 6 g of food daily. At the end of this period the amount was decreased to 5 g per day. On the 91st day one rat died in each of the groups I, II, and IV (none in the "B + supplement" group). The rats which died weighed 94, 93 and 95 g respectively. On this day all the rats were allowed their respective diets *ad libitum*. The only symptom observed by this time besides the severe emaciation

1 Burr, George O., and Burr, M. M., *J. Biol. Chem.*, 1930, **86**, 587.

2 Burr, G. O., and Burr, M. M., *J. Biol. Chem.*, 1929, **82**, 345.

3 Graham, C. E., and Griffith, W. H., Proc. Soc. Exp. Biol. and Med., 1931, **28**, 756.

4 Turpeinen, O., *J. Nutrition*, 1938, **15**, 351.

5 Hume, E. M., Nunn, L. C. A., Smedley- Maclean, I., and Smith, H. H., *Biochem. J.*, 1938, **32**, 2162.

TABLE I.
Composition of Diets.

Dietary component	I Diet A	II Diet B	III Diet B + supplement	IV Diet C
Casein (fat free)*	30.0	30.0	30.0	30.0
Sucrose	66.0	66.0	66.0	56.0
Salt mixture IV†	4.0	4.0	4.0	4.0
Dried brewer's yeast	—	—	—	10.0
		per 100 g of diet		
Thiamine HCl	.20 mg	20.00 mg	20.00 mg	—
Riboflavin	.40	40.00	40.00	—
Pyridoxine HCl	.20	2.00	2.00	—
Calcium pantothenate	.30	3.00	3.00	—
Choline chloride	120.00	120.00	120.00	120.00
3-Methyl-naphthaquinone	.50	.50	.50	.50
Biotin	—	—	.02	—
Folic acid	—	—	.20	—
Inositol	—	—	50.00	—
p-Aminobenzoic acid	—	—	15.00	—
Niacin	—	—	.60	—
Corn oil	—	—	500.00	—

* Extracted for three 2-hour periods with boiling alcohol.
† Phillips, P. H., and Hart, E. B., J. Biol. Chem., 1935, 109, 657.

was slight rustiness of fur about the shoulders and neck.

On the 107th day (16 days after the start of the *ad libitum* feeding) various skin symptoms were noticed in the rats of the first 2 groups. Among these, scaly hind paws were almost uniformly present. Other symptoms which appeared among the rats, though not uniformly, were: scaly and/or annular tail; necrosis and loss of tip of tail; sloughing off of skin near base of tail and/or around the mouth; loss of hair around the mouth, eyes, over fore paws, at the base of tail and around the scrotum; thinness of hair coat over back, chest and abdomen; some edema of fore and/or hind paws and prolaps penis. The animals receiving the diet containing yeast developed scaly paws and other skin symptoms a few days later than the first 2 groups, while the group on the purified diet which was supplemented, as mentioned above (Table I), with both additional vitamins of the B complex and with 0.5% corn oil showed practically no symptoms at any time.

When these symptoms were evident, the animals in Groups I and IV were supplemented with 0.5% corn oil and those in Group II with the extra 5 vitamins included in diet III in order to see which of these factors was involved in the prevention of the symptoms. The results of this experiment are summarized in Table II. Those animals which received additional vitamins (no corn oil) still showed scaly paws 7 weeks later, the time at which they were killed. All the rats which received the corn oil after having demonstrated the symptoms, started to show signs of recovery in 2 weeks and were completely cured within 5-6 weeks.

Along with the caloric restricted rats there was an equal number of rats on *ad libitum* regimen from the beginning of the experiment. None of these showed any symptoms at any time.

In the second experiment Diet A was used as the basal diet, and all the rats were again supplemented with one drop haliver oil weekly. The depletion period lasted 39 days, during which time the average weight dropped from 225 to 120 g. The rats were divided into two groups at this time and one group was continued on Diet A, while the other received Diet A supplemented with 0.5% corn oil. The results are summarized in Table III.

The effectiveness of corn oil in preventing development of the symptoms is obvious. The only symptoms which appeared in 5 rats of the supplemented group were slight and transient. Again the appearance of the

TABLE II.
Condition of animals at Different Periods.

	Beginning of exper.	End of depletion	Appearance of scaly paws	Beginning of disappearance of scaly paws due to ½% corn oil	Complete clearing before exper. terminated
I—Diet A					
Days	0	91	107	120	154
Total No. rats	4	3			
No. rats affected			3	3	3
%			100	100	100
Weight	223	118	153	200	334
II—Diet B					
Days	0	91	107	120	154
Total No. rats	4	3			3
No. rats affected			3		0
%			100		0
Weight	223	118	188	241	286
III—Diet B + supplement					
Days	0	91	107	120	154
Total No. rats	4	4	4	4	
No. rats affected			0		
%			0		
Weight	223	130	229	285	338
IV—Diet C					
Days	0	91	107 112	120	154
Total No. rats	4	3	3 3	3	3
No. of rats affected			0 3	3	3
%			0 100	100	100
Weight	223	125	209 238	266	317

other skin symptoms was observed in a large number of the rats on the unsupplemented diet.

Five of the rats from the unsupplemented group were given 2 drops of corn oil daily after they demonstrated the marked symptoms. Some improvement was noticed in all animals within 2 weeks, and they were all completely clear of symptoms within 3-5 weeks.

Ten rats were kept on the unsupplemented Diet A for 71 days. At the end of this period it was noticed that the scaliness of the paws had completely disappeared.

In the third experiment the animals were placed for 3 weeks on restricted amounts of our stock ration containing: ground wheat 25.00, soybean oil meal 10.00, linseed oil meal 10.00, ground yellow corn 29.00, powdered skim milk 12.00, alfalfa meal 8.00, butter 5.0, NaCl (iodized) 1.0, CaCO$_3$ 0.5. This was followed by 4 weeks on restricted amounts (5 g/day/rat) of fat free Diet A, supplemented by one drop of haliver oil weekly. At the time when *ad libitum* feeding was started the rats were divided into 4 groups

receiving Diet A with supplements listed in Table IV, plus one drop of haliver oil/week/-rat. To guard against the possibility that the ultimate disappearance of the scaly paws of the unsupplemented animals was due to very small amounts of essential fatty acids in the haliver oil fed, in this and the following experiments carotene, calciferol (D$_2$) and alpha tocopherol suspended in propylene glycol or glyceryl (mono) laurate or methyl oleate were substituted for the haliver oil at the time when the scaliness of the paws appeared.

All of the 9 animals which received no essential fatty acid showed development of scaly paws within 2 weeks after the initiation of the *ad libitum* feeding. The 10 rats which received either ethyl-linoleate or corn oil did not develop the scaly paws except for a slight scaliness in 3 animals, but this disappeared in a few days. The average weights of the animals at the commencement of *ad libitum* feeding and at subsequent periods are recorded in Table IV.

It should be noted that in the 9 animals

TABLE III.
Average Weight and Number of Rats.

Supplement	Starting *ad lib.* feeding	Developing scaly paws within 17 days	Developing scaly paws within 28 days	Clearing in 1-2 weeks	Kept for 71 days	Cleared up spontaneously within 71 days
None	31 (126)*	26 (166)	31 (230)	0	10	10
0.5% corn oil	11 (129)	5 (slight) (182)	— (230)	5	—	—

* The numbers in parentheses indicate average weight.

TABLE IV.
Average Weight and Number of Rats.

Supplements	In a group starting *ad lib.*	Developing scaly paws within 2 weeks and lasting longer than 1 week	Showing scaly paws after 6 weeks	Showing complete clearance at 66 days
None	4 (114)*	4 (166)	4 (224)	4
2 drops oleic acid daily	5 (116)	5 (187)	5 (251)	5
2 drops ethyl linoleate daily	5 (117)	0 (178)	0 (268)	—
2 drops corn oil daily	5 (117)	0 (178)	0 (274)	—

* The numbers in parentheses indicate average weight.

which received no source of linoleic acid scaliness of the paws disappeared spontaneously, and within 66 days from the start of *ad libitum* feeding these animals, all of which had shown this symptom, were completely cured. The other skin lesions, when present, cleared much earlier.

This spontaneous disappearance of skin lesions within several weeks and paw scaliness within 66 days was again observed in a similar experiment carried out later with approximately the same number of animals on fat free ration A.

Discussion. Burr and Burr[1] stress the importance of scaliness of the feet as the most sensitive test of the essential fatty acid deficiency condition. Hume, Nunn, Smedley-Maclean and Smith[5] observed dryness occurring invariably on the dorsal surface of the hind feet and front of the ankles, and therefore they finally adopted it as the most satisfactory criterion, rather than relying entirely upon weight increase which is a non-specific response. The objection to the use of skin symptoms as criterion because of their irregular appearance[6] which is due to variation in relative humidity[7] is obviated

in our work by the constant 50% relative humidity kept in our animal room.

Although the rats in the first experiment were maintained for a long period (3 months) on a restricted intake of fat free diets, none of the symptoms described above appeared before about 2 weeks after *ad libitum* feeding commenced, a period during which there was rapid regain of weight. In later experiments, the depletion periods on the fat free diet were considerably shorter (4-6 weeks), yet the symptoms again appeared within 2-3 weeks after the beginning of the *ad libitum* regimen.

The typical symptoms of essential fatty acid deficiency were produced heretofore only when young rats were used and generally after very long periods of fat deprivation,[8,9] yet in our work the mature rats demonstrated the symptoms after a total period of 6-7 weeks

6 Evans, H. M., and Lepkovsky, S., *J. Biol. Chem.*, 1932, 96, 587.

7 Brown, W. R., and Burr, G. O., *Proc. Am. Soc. Biol. Chem.*, 1936, 8, XVI.

8 Hume, E. M., and Smith, H. H., *Biochem. J.*, 1931, 25, 300.

9 Gregory, E., and Drumund, J. C. Z., *Vitaminforschung*, 1932, 1, 257.

on a fat free diet. Therefore, the technique seems to suggest itself for other types of experiments where a rapid "growth" (weight gain) or some "strain" on body functions is desired.

The ultimate disappearance of the scaly paws in these mature rats may be explained on the basis of: (1) lower demand for essential fatty acids when the rate of tissue building reaches normal maintenance level, (2) synthesis of at least a limited amount of an "essential" fatty acid within the animal itself, or (3) the combined effect of 1 and 2 in these mature animals.

Smedley-Maclean and Hume[10] found that the polyunsaturated acid content of the tissue of fat starved rats fell to a very low level during the first 6 months of fat starvation. In our rats the total amounts of essential fatty acids in the emaciated bodies at the commencement of *ad libitum* feeding is probably very low. Smedley-Maclean and Nunn[11] concluded that in the process of growth comparatively large quantities of arachidonic acid are utilized and disappear. In the light of these findings and the observed weight gains in our rats with the beginning of the *ad libitum* feeding of the fat free diet it seems likely that they further depleted their supply of essential fatty acids when the scaliness of the paws appeared. After this

there was further weight gain in which essential fatty acids were further utilized, yet the scaly symptoms finally disappeared without any external source of these acids. Therefore, it seems likely that at least a limited amount of the essential fatty acids is synthesized in the bodies of these mature rats. This appears to be in agreement with the work of Harris[12] who used the deuterium technique and found that under appropriate conditions the rat can synthesize the essential fatty acid arachidonic acid.

Summary. 1. Mature rats were maintained on a fat free diet for relatively long periods without showing any deficiency symptoms except emaciation.

2. Severe depletion followed by *ad libitum* feeding of the fat free diet precipitated in mature rats symptoms including those typical of essential fatty acid deficiency.

3. This method is suggested for other types of experiments where a "strain" on body functions is desired.

4. Either ethyl linoleate or corn oil prevented entirely or cured rapidly the symptoms of essential fatty acid deficiency when they appeared.

5. When the rats, after depletion, were maintained on the fat free diet *ad libitum* for sufficiently long periods, spontaneous disappearance of all the symptoms was observed, for which the explanation of some synthesis of essential fatty acid is suggested.

10 Smedley-Maclean, I., and Hume, E. M., *Biochem. J.*, 1941, **35**, 990.

11 Smedley-Maclean, I., and Nunn, L. C. A., *Biochem. J.*, 1940, **34**, 884.

12 Harris, R. S., 24th Annual Report of the National Live Stock and Meat Board, 1946-47.

16129

Effect of Carbon Dioxide and Other Gases on Electrocardiogram of the Right Ventricle.[*]

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It has been reported[1,2] that the intravenous injection of carbon dioxide causes the formation of a gas bubble in the right ventricle with a concomitant lowering of the initial ventricular deflection of the electrocardiogram when this is recorded directly from the surface of the right ventricle. Further investigation has shown that this electrical phenomenon occurs not only with carbon dioxide, as was first thought[1,2] but also with the parenteral injection of other gases.

Procedure. Mongrel dogs, averaging 6-12 kilos in weight, were anesthetized with sodium pentobarbital (approx. 35 mg/kilo) by vein. Respirations were maintained through a tracheotomy tube after the chest had been opened with a sternum-splitting incision. In addition to the standard electrocardiographic leads, direct leads were taken by placing a wick electrode without pressure on the heart muscle through a small slit in the pericardium. The various gases were injected through an exposed vein.

The electrocardiograms were taken with a Cambridge Simplitrol, using a fast speed (50 mm/sec.), a thick string casting a 3 mm shadow, and increased intensity of the light source (using a variac transformer). These three improvements made the elements of the QRS complex of the electrocardiogram much easier to read.

Results. As has already been described[1,2] when 50-100 cc of a gas is rapidly injected into the femoral vein of a small dog (6-10 kilos), lying supine, the gas collects in the right ventricle, blocking the pulmonary conus and artery, preventing the passage of blood into the lungs. The right heart dilates; the blood pressure falls. If the gas is carbon dioxide, which is quite soluble, it is readily absorbed by the blood in 15-17 seconds. Circulation through the lungs is resumed as the bubble is reabsorbed and the dog recovers. Other gases or mixtures of gases, such as oxygen, nitrogen, helium and air, are not so readily absorbed, and if the dog's position is not changed, death will occur in 3-5 minutes because of the complete block to the pulmonary circulation. If the gas bubble is not large, the pulmonary circulation may be reestablished by turning the animal into the left lateral position.[3] The gas bubble then floats up from the pulmonary conus to the apex of the right ventricle and allows blood to flow into the pulmonary artery.

As the gas bubble dilates the right ventricle an interesting electrocardiographic change takes place. The initial upward deflection of the electrocardiogram in a direct lead from the right ventricle is lowered or disappears entirely. If only a small amount of gas (10-15 cc) such as carbon dioxide is used, the R wave is merely lowered. With 50 cc or more of carbon dioxide the R wave disappears entirely (Fig. 1). At the same time the P wave increases in amplitude and the ST segment may be slightly raised, with inversion of the T wave. There is no change in the PR interval or in the width of the QRS complex. As the carbon dioxide is absorbed the R wave reappears and the electrocardiogram assumes its control appearance. Repeated injections of CO_2 produce the same sequence of events.

In fatal experiments with the less soluble

[*] Supported in part by RG 194, N.I.H.

[1] Durant, T., Long, J., and Oppenheimer, M. J., *Am. J. Med. Sc.*, 1947, **213**, 633.

[2] Durant, T., Long, J., and Oppenheimer, M. J., *Fed. Proc.*, 1947, **6**, 435.

[3] Durant, T. M., Long, J., and Oppenheimer, M. J., *Am. Heart J.*, 1947, **33**, 269.
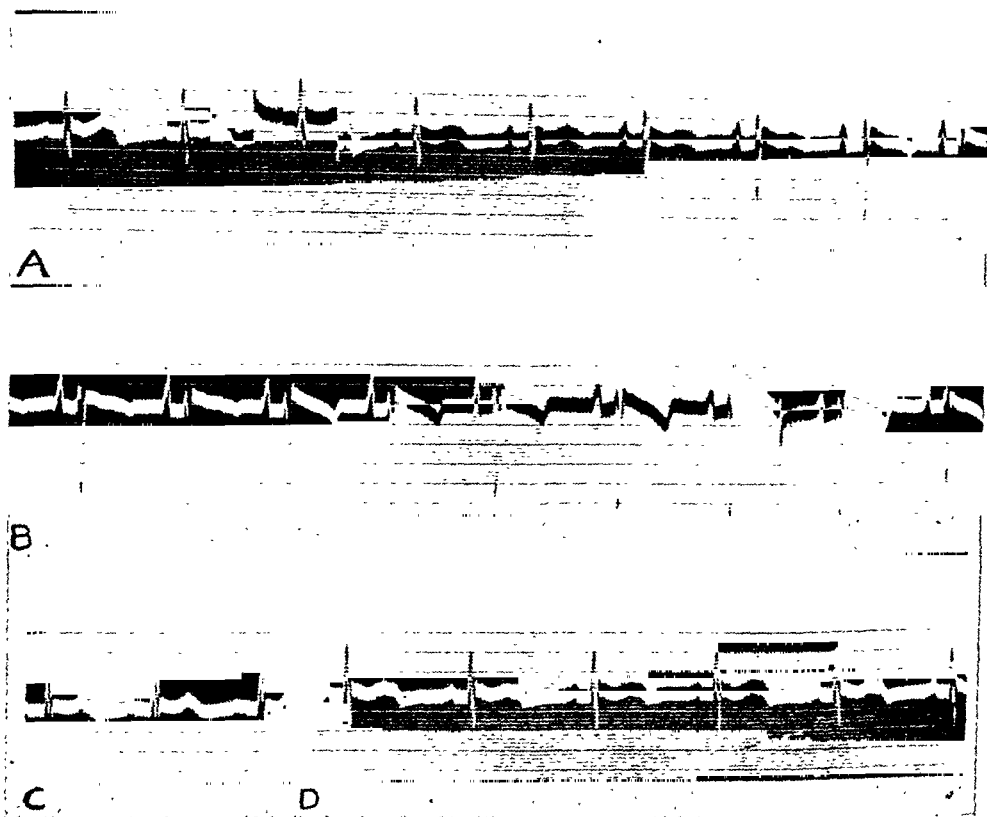
FIG. 1.

Male dog, 8.2 kg 9/16/47. A. Two control complexes and beginning of injection of CO_2 at millivolt signal (3 millivolts). B. Continuation of A with no complexes omitted. End of injection at millivolt signal. C. Between the end of B and the beginning of C 5 minutes elapsed. D. Between C and D 5 minutes have elapsed. Anesthesia: Pentobarbital sodium, 35 mg/kg. Camera speed 50 mm/sec. Direct lead from right ventricle.

gases the R wave disappears and may not reappear. In addition the ST segment and the T wave changes become gradually more pronounced.[3]

The etiology of the change in the QRS complex is of interest. It is known that interposing an insulating substance such as air in hyperdistended lungs between the heart and the exploring electrode of chest leads will cause a lowering of the voltage of the R wave.[4,5] Katz and co-workers[6] showed that when parts of the heart surface are short-circuited by means of lead or tinfoil or insulated by glass or rubber, that the voltage of the electrocardiogram is decreased. Any such etiology is ruled out in these experiments because the exploring electrode is directly on the heart muscle.

Another possible cause of the electrocardiographic change is the anoxia caused by the interruption of the blood flow through the lungs. Harris and Randall[4] showed that the reduction of the height of the R wave in anoxic dogs with closed chests was associated with the enlargement of the thorax as a result of the hyperpnea and that there was no change in the height of the R wave in direct leads from the heart in dogs with open chests in concentrations of oxygen as low as 7%.

When animals in our laboratory were sub-

4 Harris, A. S., and Randall, W. C., Am. J. Physiol., 1944, 142, 452.

5 Lapin, A. W., Am. Heart J., 1947, 33, 747.

6 Katz, L. and Korey, H., Am. J. Physiol., 1935, 111, 83.
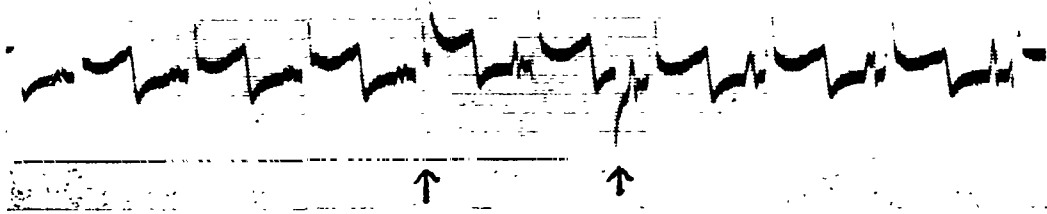
FIG. 2.
Male dog, 8.0 kg 9/30/47. Three control complexes and beginning of mineral oil injection at millivolt signal (3 millivolts). Anesthesia: Pentobarbital sodium, 35 mg/kg. Camera speed, 50 mm/sec. Direct lead from right ventricle. R_1 and R_2 are present in the control complexes. R_1 alone is diminished by the injection of mineral oil. The two arrows indicate the duration of the millivolt signal.

jected to asphyxia by cutting off the artificial respiration, the right heart dilated but there was no lowering of R.

Sudden dilatation of the right ventricle by means of the rapid injection of 100 cc of blood into the jugular vein or by compressing the pulmonary artery was found to be ineffective in changing the amplitude of the R wave.

An important possibility to consider is that the electrocardiographic changes are due to the replacement of blood in the right ventricular cavity by a dielectric. It was found that when mineral oil, a non-conducting liquid, was injected rapidly in sufficient quantities to completely replace the blood in the right ventricle (50 to 100 cc in a 10-kilo dog) the same phenomenon occurred, i.e. there was a marked lowering or disappearance of the R wave (Fig. 2). In each case a post-mortem check of the contents of the ventricle was made. In one experiment, wherein only 50 cc of oil was injected in a 12-kilo dog, the R wave was unchanged, and at post-mortem

the right ventricle was found to contain some oil mixed with a large amount of blood, hence complete replacement by a dielectric had not occurred.

The injection of carbon dioxide into the left ventricular cavity in several experiments resulted in the production of multiple extrasystoles, making it impossible to evaluate its effect on the R wave in leads from the left ventricle. Moreover, we have been unable to adequately fill the left ventricle with mineral oil so as to study the effects of this dielectric on left ventricular leads. Furthermore, these experiments are complicated by the effects of coronary artery filling by the injected substance.

Conclusion. The rapid intravenous injection of gases or mineral oil in sufficient quantities to replace blood in the right ventricle of dogs will cause the reduction or loss of the initial ventricular deflection of the electrocardiogram in direct leads from the epicardial surface of the right ventricle.

16130

Methionine Excretion. Effect of Diet and Methionine Ingestion in Normal Subjects.

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A number of reports[1] in the literature have indicated the outstandingly important role that methionine may play in nitrogen metabolism. Its utilization has been shown to involve not only the intact amino acid but also the sulfur and labile methyl groups in the formation of other essential substances. The varied uses of methionine have been shown by its lipotropic activity and protein sparing action in animals on very low protein diets, and by its role in the detoxification of a variety of organic compounds.[2]

The recently emphasized physiological importance of methionine, along with our earlier studies[3] on animals with fatty livers produced by diets deficient in this amino acid, suggested an investigation of methionine excretion as a new approach to the study of methionine metabolism and as a possible indication of its utilization.[4] Changes in urinary methionine excretion might be caused by increased requirements for this amino acid for essential body proteins. Also increased requirements, which could not be met from available methionine, might be reflected by a decreased excretory response to a test supplement.

Information about the excretion of methionine by normal individuals is scanty. Cleland,[5] in her study of methionine excretion in cystinuria, obtained normal values of the same order as those found by Albanese and coworkers,[4,6] and they both reported an immediate rise in its excretion after ingestion of the free amino acid. If an increase in the protein intake should cause a rise in the excretion of methionine as in the cystinuric,[5] then the effect of fasting and of various dietary regimes, such as low protein, higher protein, and high fat diets, upon its urinary loss should be considered in excretion studies.

Methods. The subjects used in this study were 80 male medical students. The urinary samples for methionine analysis were collected over the 6-hour period, 7 a. m. to 1 p. m., and for the subsequent 18 hours when 24-hour collections were desired. The 6-hour collections were included as a more convenient period of measurement since Albanese[4] has shown a return to the normal level of excretion 3 hours after an intake of 1.5 g of methionine, with equal utilization of the natural and racemic forms. Usually on the following day at 7 a. m., the subjects, fed or fasted, were given 1.0 or 1.5 g of *dl*-methionine orally. The urine collections were started at that time and completed at the end of the same periods used for the controls.

In a second experiment, low protein, higher protein, and high fat diets were followed for 4 days by selecting food from lists of items suitable for such diets. Urine collections were made on the last day of the special diets. Besides methionine, total nitrogen and pH values were determined on these urine samples to indicate how well the diets had been followed. The subjects on high fat diet ingested 20% cream which contains about 2.5 times more ketogenic than antiketogenic substances. This accounts for the fair intake of methionine and only traces of acetone in the urine. They

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1 Lugg, J. W. H., *Ann. Rev. Biochem., Stanford University*, 1945, 14, 271.

2 Miller, L. L., *J. Biol. Chem.*, 1944, 152, 603. Also references 1 and 5-7.

3 Treadwell, C. R., Tidwell, H. C., and Gast, J. H., *J. Biol. Chem.*, 1944, 156, 237.

4 Albanese, A. A., *Bull. Johns Hopkins Hosp.*, 1944, 75, 175.

5 Cleland, J. B., *Australian J. Exp. Biol. and Med. Science*, 1945, 23, 273.

6 Albanese, A. A., Frankston, J. E., and Irby, V., *J. Biol. Chem.*, 1944, 156, 293.

were given a supplement of 1.5 g of methionine and only the 6-hour collections were examined. Separate normal excretion values were obtained on these subjects at this time.

The second experiment was repeated in part on a similar group of students one year later. In addition, 24-hour urine collections, after fasting and a high fat diet, were obtained for examination. Both of these groups developed a marked ketonuria.

In all these studies, total nitrogen excretion values were used to calculate the protein intake of the subjects on the various diets, after allowing 3 days on the diet for stabilizing the plane of nitrogen metabolism. Methionine intakes were estimated by assuming that the protein of the diet contained an average of 3% methionine.[7] The urinary methionine was determined according to the method of Albanese et al.[4] Quantitative recovery of methionine added to the urine by us indicates the suitability of the method for the estimation of methionine in urine. However, it must be remembered that the method will respond to any other unknown terminal thio ether or thiocarbonyl group containing substances of the urine. The data were analyzed according to the t method of Fisher[8] and only those results showing a P value of 0.01 or less were considered significant. The standard error of the mean for each value is given with the data.

Results. Our results on the daily urinary excretion of methionine by the adult male compare favorably with those of Albanese et al.[4] and Cleland.[5] The former group found an excretion of 247 to 494 mg of methionine per day in a series of 7 observations. Using 80 different subjects, we obtained an average daily excretion of 318 ± 9 mg (range 199 to 518 mg) or about 4.5 mg per kg body weight.

The methionine excretion during 6- and 24-hour periods and following the ingestion of a 1.0 or 1.5 g supplement is shown in Table I. The latter supplement was approximately one-half of Block's estimate[9] of the average daily intake of methionine. An increased amount of methionine was excreted after the one g test supplement and definitely more after the 1.5 g. However, the percentage of the supplement excreted was the same in both cases, approximately 9% in 6 hours and 15% in 24 hours respectively. An overnight fast caused only a slight change in the methionine excreted, either in the absolute amount of the amino acid, or in the per cent of the test supplement given. Thus, if excretion be taken as a measure, the major part of the free amino acid was utilized (stored or metabolized) even when the intake was markedly increased by the additional methionine ingested.

The remainder of this study was concerned with the effect of fasting and of various diets upon the daily methionine excretion in the normal subject. Three types of diet were ingested—low protein, higher protein, and high fat diets. The total nitrogen of the urines of subjects on these diets served as a check upon the protein intake. Averages of 6.5, 17.7, and 9.3 g of total urinary nitrogen per day, respectively, were obtained in the second experiment. The pH of the urines, the methionine intakes and amounts excreted are included in Table II. Similar data for the third experiment are shown in Table III.

There was no significant difference in the final 24-hour urinary methionine excretions by the subjects on the low and higher protein diets for a period of 4 days. This was true in both experiments on humans (Table II and III), and the observation has been confirmed with rats.[10] These results are supported by the relative constancy of the neutral sulfur excretion.[11] Thus marked variations in the protein content of the diet,† did not affect the daily urinary loss of methionine in these

[7] Block, R. J., and Bolling, D., The Amino Acid Composition of Proteins and Natural Foods, C. C. Thomas, Springfield, Ill., 1944.

[8] Fisher, R. A., Statistical Methods for Research Workers, 7th ed., Oliver and Boyd, Edinburgh, 1938.

[9] Block, R. J., *Yale J. Biol. Med.*, 1943, **15**, 723.

[10] Tidwell, H. C., unpublished data.

[11] Deuel, H. J., Jr., Sandiford, I., Sandiford, K., and Boothby, W. M., *J. Biol. Chem.*, 1928, **76**, 391.

† Since completion of these studies, a similar observation has been reported by Johnson, R. M., Deuel, H. J., Jr., Morehouse, M. G., and Mehl, J. W., *J. Nutrition*, 1947, **33**, 371.

TABLE I.
Effect of Methionine Ingestion upon Its Excretion.

The fed subjects ingested their normal diets at the usual hours while the fasted subjects had no food for 18 hours including the 6 hours of the urine collection. The normal diets contained approximately 2 g methionine per day.

			Methionine excreted			
			6 hr		24 hr	
No. of subjects	Nutritional state	Methionine supplement, g	total, mg	per kg, mg	total, mg	per kg, mg
11	Fed	0.0	99	1.41	406	5.79
			± 5	±0.07	±13	±0.02
11	,,	1.0	185	2.65	550	7.85
			±11	±0.16	±18	±0.28
15	,,	0.0	98	1.37	415*	5.73*
			± 5	±0.20	±32	±0.46
15	,,	1.5	229	3.16	665*	9.34*
			± 9	±0.15	±41	±0.46
11	Fasted	0.0	83	1.15		
			± 5	±0.07		
11	,,	1.5	206	2.85		
			± 7	±0.18		

* Includes 8 subjects.

TABLE II.
Effect of Diet on Urinary Methionine Excretion.

Exp. 2. Subjects ingested the special diets for 4 days. Determinations were made on samples collected during the 6 or 24 hours of the last day. Daily methionine intake was estimated by assuming that the protein of the diet contained 3% methionine.[7]

			Calculated methionine intake, g/day	Methionine excreted	
No. of subjects	Dietary regime	Urine pH		total, mg	per kg, mg
12	Low protein	6.2	1.22	267	3.75
		±0.2	±0.11	±15	±0.19
9	Higher protein	5.6	3.32	252	3.52
		±0.1	±0.06	±19	±0.24
28	Average			257	3.62
				±11	±0.13
9	Normal	6.5	2.10	85†	1.24†
		±0.3		± 5	±0.14
9	,, *		2.10	223†	3.11†
			±0.15	±11	±0.15
11	High fat*	5.0	1.74	154†	2.25†
		±0.1	±0.13	± 9	±0.13

* After a 1.5 g test supplement of methionine.
† Determinations made on 6-hr collections.

subjects. The increased methionine excretion after the ingestion of the free amino acid as compared with that after its ingestion in the form of protein might be explained by an increased rate of absorption from the gastrointestinal tract. Fratzer[12] found that the oral administration of hydrolyzed protein produced a greater increase in the blood amino acid level than did the ingested intact protein,

and that methionine caused higher levels than did some of the other amino acids. The higher blood levels might account for the increased methionine excretion after the ingestion of the free amino acid.

Although a marked change in the protein intake was without effect on the urinary methionine excretion, a 3-day fast or high fat diet was associated with a significantly decreased excretion as compared with that on

12 Fratzer, F. H., J. Biol. Chem., 1944, 153, 237.

TABLE III.
Effect of Diet on Urinary Methionine Excretion.
Exp. 3. Determinations were made on 24-hour samples of the fourth day on these dietary regimes. Daily methionine intake calculated as in Table II.

No. of subjects	Dietary regime	Urine pH	Total N excreted, g/day	Calculated methionine intake, g/day	Methionine excreted	
					total, mg	per kg, mg
13	Low protein	6.6	7.15	1.34	298	4.18
		±0.1	±0.34	±0.07	±14	±0.20
12	Higher protein	6.0	17.74	3.33	290	4.21
		±0.1	±1.29	±0.24	±12	±0.26
12	High fat	5.7	11.88	2.23	252	3.48
		±0.2	±0.97	±0.18	±30	±0.43
9	Fasting	5.1	11.95	0	269	3.42
		±0.0	±0.74		±26	±0.39

the low and higher protein diets used. Also, there was a marked decrease in the urinary methionine after a test supplement was given the subjects on the high fat diet as compared with the normal control values as shown in Table II. Circumstances prevented 6-hour high fat control values on these subjects, but a decreased loss of methionine was found in a 24-hour test in a similar group in the third experiment. Hence a lowered base level of excretion, and a possible slower rate of absorption, might account for a part of the decreased excretion of the supplement in these subjects. However, it is evident that a very high fat intake or fasting, accompanied by a ketonuria, was associated with a decrease in methionine excretion. Increased needs for methionine for lipotropic requirements at times of excessive fat metabolism, owing to a very high fat intake or the excessive use of body fat during fasting, might account for the decreased excretion in these subjects. In this case greater body needs appear to be followed by a decreased excretory response.

The excretion of less methionine by the subjects on the special diets as compared with that of the others in this study was associated with smaller urine volumes during warmer weather. The urines for the dietary studies were collected around May 30 when the average temperature was above 80°F, while the other values were obtained during February and March with temperatures averaging near 60°F. Lack of other known variables than the temperature suggested possible losses of methionine through channels other than

the kidneys. Nevertheless, we were unable to correlate the daily urine volumes and the urinary methionine excretion in the various experiments.

Another interesting fact was noted when the per cent retentions of methionine on the different protein intakes in the last two experiments were compared. Values of 78% retention on the low, and 92% on the higher protein diets were almost identical in the last two experiments. This decreased loss in the latter case suggests an increased utilization of methionine with the higher protein intakes.

Summary. Observations on a series of 80 normal male subjects, on a normal control diet, indicated an average daily urinary excretion of approximately 318 ± 9 mg of methionine. The increase in methionine excretion during the 6- and 24-hour periods following the ingestion of a 1.0 or 1.5 g supplement of methionine in addition to that of the usual protein intake only amounted to approximately 9 and 15% respectively of the intake of the free amino acid.

The ingestion for 4 days of diets of varying protein content showed no change in the daily urinary methionine excretion as a result of such variation. A decrease in the methionine normally excreted occurred when the subjects were on a high fat diet or when, on a 6-hour test, they were given a similar test supplement of methionine. Increased lipotropic requirements might account for the decrease in the loss of methionine after a 3-day fast or high fat intake.

16131

An Acoustical Indicator of the Systolic End Point in Rat Blood Pressure Determinations.*

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An electrical device for determining the systolic end point of rat blood pressures has been described by Skeggs and Leonards.[1] It consists of a small carbon telephone transmitter button placed in series with an ammeter in a low voltage wheatstone bridge circuit. Pressure on the button decreases its resistance resulting in deflection of an ammeter needle. Pressure is transmitted from the tail to the button by means of a simple lever system. Engorgement of the tail vessels and increase of size with pressure on the button occur at the systolic blood pressure level following release of occlusion of the base of the tail by an air cuff. At the moment of decrease in electrical resistance and sweep of the ammeter needle, blood pressure is read from a manometer in the air cuff system.

This electrical end point device of Skeggs and Leonards requires alternate visual observation of an ammeter needle and of a sphygmomanometer. The modification to be described was devised to facilitate and improve accuracy of readings by permitting simultaneous visual and auditory observation. A set of headphones and an amplifier circuit was substituted for the ammeter. Decrease of resistance within the carbon button at the end point is heard as a change of tone permitting undivided visual attention to the sphygmomanometer.

Acoustical Indicator. The circuit for the acoustical indicator is illustrated in Fig. 1. A sensitive carbon button similar to that of the Skeggs and Leonards apparatus is placed in the grid circuit of a 1LE3 triode amplifier tube. The neon lamp employed is of low wattage ($\frac{1}{4}$ watt) and has no internal resistor. The fixed condenser has a capacity of

0.002 Mfd. A variable resistor of 5,000 ohms is placed in series with the carbon button and another variable resistor of 7,500 ohms is put in series with the cathode and the neon lamp. A fixed carbon resistor of 270,000 ohms is required in series with the plate of the triode tube. The headphones have a resistance of 3,000 ohms.

The circuit operates on direct current from batteries. A switch is provided to disconnect all batteries except the 120 v power supply when the apparatus is not in use. This circuit produces a rapidly interrupted current audible in the headphones as a distinct tone. The tone can be varied to a higher or lower pitch by changing the two variable resistors permitting adjustment for maximum change of pitch when engorgement of the vessels of the rat's tail produces pressure on the carbon button.

Method of Use. The technique of taking rat blood pressures with the acoustical indicator is similar to that used when employing the ammeter circuit. The rat tail is adjusted in the carbon button holder and then the acoustical indicator is switched on. The pressure in the air cuff is elevated above the expected blood pressure and then slowly released. The tone in the headphones rises during inflation of the cuff and decreases with release of the cuff pressure until the systolic end point is reached when the pitch again increases. The end point is indicated by a smooth, rapid increase in pitch which, after attaining its maximum height, will be maintained until the cuff pressure is lowered 20 to 70 mm Hg below the systolic pressure. Artifacts produced by movements of the tail are distinguishable as rapid, transient changes in pitch.

Unfortunately it has not been possible to obtain blood pressure readings without producing vasodilatation of the tail by preparing the rat. The conventional method of obtaining

1 Skeggs, L. T. and Leonards, J. R., PROC. SOC. EXP. BIOL. AND MED., 1946, 63, 294.
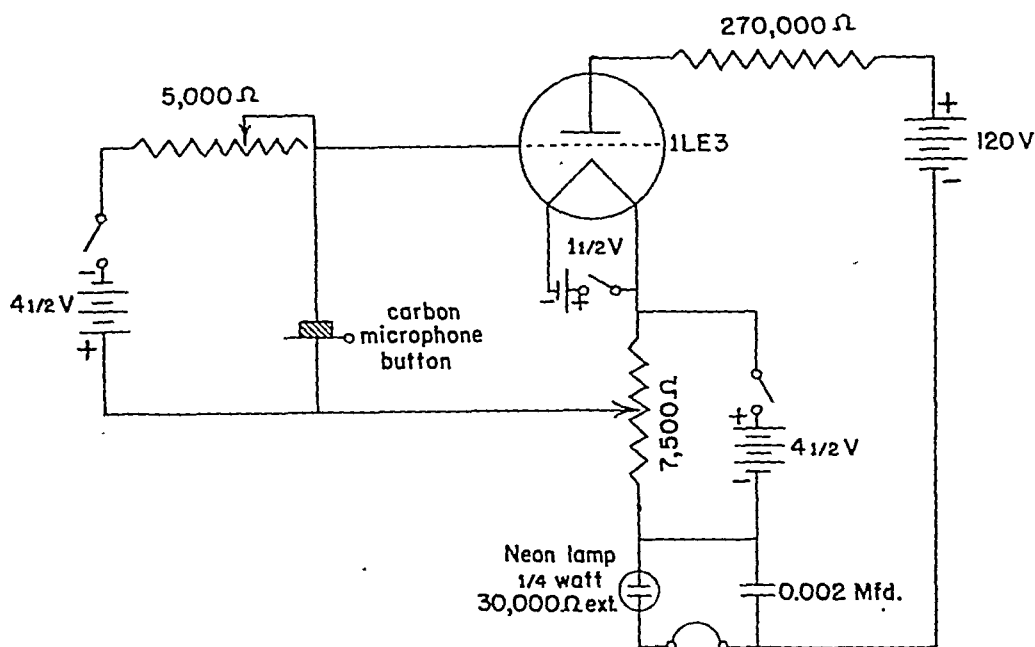
Fig. 1

vasodilatation is heating of the entire rat or of the tail alone. Williams, Harrison, and Grollman[2] have reported heating of the entire rat, exposing it to 40-42°C for 10 minutes before obtaining readings. They used the plethysmograph technique. Sobin[3] has reported equal success after heating the tail alone. Proskauer, Neumann, and Graef[4] have reported that heating elevates rat blood pressure. In our experience heating of the entire animal at 40-42°C for 10 minutes increased the rectal temperature on the average 3.3° Heating the tail alone at 42-45°C increased the rectal temperature on the average 1.9°C. However, heating either the entire animal or the tail alone produced comparable readings of systolic blood pressure.

Another method of obtaining readings is the use of a peripheral vasodilating agent. 2-Benzyl-4, 5-Imidazoline HCl (Priscol) dilates the tail vessels of the rat and permits determination of systolic blood pressure without increase of temperature of the rat. Ad-

ministration of 3-4 mg/kg Priscol intramuscularly permitted blood pressure readings after 45 to 60 minutes which could be repeated at will during a period up to 9 or 10 hours after the drug.

Either heat to the entire rat or Priscol was used for the experiments below comparing 3 methods for blood pressure determinations, Grollman (plethysmographic), Skeggs and Leonards, and the acoustical modification.

Experimental Evaluation. Readings of the rat systolic blood pressure were compared using the Grollman plethysmograph technique, the ammeter circuit, and the acoustical indicator. (Table I). Simultaneous readings were obtained with the plethysmograph and one or the other electrical end point devices. Readings were repeated alternating the electrical methods thereby comparing all 3 methods in 6 to 7 minutes. Plethysmographic readings were consistently lower than obtained with either electrical device. The average difference in blood pressure was 3-5 mm Hg with heated rats and 9-10 mm Hg with unheated Priscol treated rats. Both electrical end point devices would often give blood pressure readings following Priscol preparation when they were unobtainable with the

[2] Williams, J. R., Jr., Harrison, T. R., and Grollman, A., *J. Clin. Invest.*, 1939, **18**, 373.

[3] Sobin, S. S., *Am. J. Physiol.*, 1946, **146**, 179.

[4] Proskauer, G. G., Neumann, D., and Graef, I., *Am. J. Physiol.*, 1945, **143**, 290.

TABLE I.

Comparison of Systolic Blood Pressure Readings by 3 Methods in Heated Normal Rats and in Priscol Treated Rats Without Heating.

Rat	Rats heated 10 min. at 40°-41°C				Priscol treated		
	Plethysmograph	Ammeter	Headphones		Plethysmograph	Ammeter	Headphones
1	108	112		*	90		104
	112	110			90		94
	112	114			88		92
	104		114		90	110	
	106		108		100	106	
	110		116		98	102	
2	98		102	*	100	112	
	98		98		98	104	
	96		96		90	108	
	90	90			96		102
	90	90			94		102
	88	88			90		98
3	116		122	†	70		88
	114		118		78		92
	114		120		84		104
	118	122			90	94	
	116	116			86	96	
	112	116			82	90	
4	84	88		†	80	94	
	82	84			90	90	
	84	84			86	92	
	78		82		82		96
	80		84		84		96
	78		82		80		94

* 3 mg/kg Priscol I.M.   † 4 mg/kg Priscol I.M.

plethysmograph. This greater sensitivity was observed during the periods of minimum vaso-dilatation before and after the maximum effect. Frequently readings taken by the ammeter circuit and the acoustical indicator coincided. However, the acoustical indicator gave readings that averaged 1.25 mm Hg above those of the ammeter circuit when a series of 100 readings by each device was compared.

Conclusion. The acoustical modification of the Skeggs and Leonards apparatus described facilitates determination of the blood pressure of rats but can be used only after vaso-dilatation produced by heat or drugs. Priscol produces vasodilatation sufficient for repeated blood pressure determination during a 9- or 10-hour period.

We wish to express appreciation to A. A. Foster for directing our attention to the electrical circuit herein described and to L. T. Skeggs and J. R. Leonards for one of their electrical end point devices.

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Relaxin Content of Blood, Urine and Other Tissues of Pregnant and Postpartum Guinea Pigs.*

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Relaxin was first detected in the blood of pregnant rabbits and guinea pigs (Hisaw[1])

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and was subsequently found in the blood of pregnant sows, dogs, cats, mares and women (Hisaw;[2] Brouha and Simonnet;[3] Pommerenke;[4] Abramson, Hurwitt and Lesnick[5]). Separation of the pubic bones which occurs

[1] Hisaw, F. L., PROC. SOC. EXP. BIOL. AND MED., 1926, 23, 661.

during the normal gestation in the guinea pig has been described by a number of investigators and this reaction, as induced experimentally by relaxin, has been used extensively in the assay of this hormone. Nevertheless, except for the rabbit (Marder and Money[6]) no attempt has been made to determine the amount of relaxin present throughout the various stages of pregnancy. Therefore, it was felt desirable to study quantitatively the concentration of relaxin in the blood, urine, uterus and placenta of guinea pigs at different times of pregnancy and after delivery of the young.

Methods. Adult female guinea pigs weighing from 600 to 800 g were mated and pregnancy dated from the morning on which the presence of vaginal plugs was noted. The average length of pregnancy was considered to be 68 days (Young and Blandau[7]). Blood was obtained from the animals by cardiac puncture at definite intervals after mating and the blood serum from a group of guinea pigs of a similar length of pregnancy pooled in order to obtain a sufficient quantity to carry out relaxin determinations. Urine samples were secured from a number of guinea pigs for a 24-hour period prior to obtaining the blood and dialyzed against running tap water. Placentæ and uteri were removed from animals on the 56th and 63rd day of pregnancy, ground up and extracted twice with 3% HCl. The extracts were kept in the cold at a pH of approximately 0.5 and prior to injection were brought to pH 7 with 10% NaOH.

The assays for relaxin content were performed according to the method of Abramo-

witz *et al.*[8] except that the guinea pigs were pretreated with one μg of estradiol daily for 3 days. Each sample was tested at several dilutions in order to abtain a concentration that gave a 67% response. The guinea pigs used for the relaxin determinations were part of a large colony maintained solely for assay purposes. In addition to the animals employed in the assay a total of 80 guinea pigs were used in this investigation, 57 for the study of pregnancy and 23 following parturition. The relaxin content of the blood serum was determined at 15, 21, 28, 35, 42, 49, 56, and 63 days after mating and at 2, 6, 24, and 48 hours postpartum. Twenty-four-hour samples of urine were obtained 42, 56, and 63 days after mating and the uterine and placental tissues were removed at 56 and 63 days of pregnancy.

Results. Relaxin assays on the 15th day of pregnancy were negative at a level of 10 ml of serum. However, a positive response was obtained by the 21st day indicating approximately 0.25 G.P. units per ml. (Fig. 1). Between the 21st day and the 28th day the concentration of relaxin was doubled, reaching a maximum value of 0.5 G.P. units per ml of serum. This level was maintained for 4 weeks and was followed by a drop to 0.33 G.P. units per ml by the 63rd day of pregnancy. Shortly after parturition a second and more precipitous decrease took place so that in the 48-hour postpartum guinea pig relaxin was no longer detectable in 10 ml of blood serum.

The urine showed an activity of 0.5 G.P. units of relaxin per ml at 42 and 56 days of pregnancy, and approximately 0.25 G.P. units per ml of urine on the 63rd day. It is of interest to note that the drop in the relaxin concentration of the urine occurred at the same time as that in the blood serum. The total volume of urine for a 24-hour period varied from 10 to 20 ml so that the guinea pigs excreted in their urine from 5 to 10 G.P. units of relaxin daily between the 28th and 56th days of pregnancy.

Approximately 10 G.P. units of relaxin per gram of fresh tissue was found in the uterus of the guinea pigs on the 56th day of pregnancy whereas the placenta showed a

2 Hisaw, F. L., *Physiol. Zool.*, 1929, **2**, 59.

3 Brouha, L., and Simonnet, H., *Compt. rend. Soc. de biol.*, 1928, **99**, 1769.

4 Pommerenke, W. T., *Am. J. Obst. and Gynecol.*, 1934, **27**, 708.

5 Abramson, D., Hurwitt, D., and Lesnick, G., *Surg. Gynec. and Obst.*, 1937, **65**, 335.

6 Marder, S. N., and Money, W. L., *Endocrinol.*, 1944, **34**, 115.

7 Young, W. C., and Blandau, R. J., *Science*, 1936, **84**, 270.

8 Abramowitz, A. A., Money, W. L., Zarrow, M. X., Talmage, R. V. N., Kleinholz, L. H., and Hisaw, F. L., *Endocrinol.*, 1944, **34**, 103.
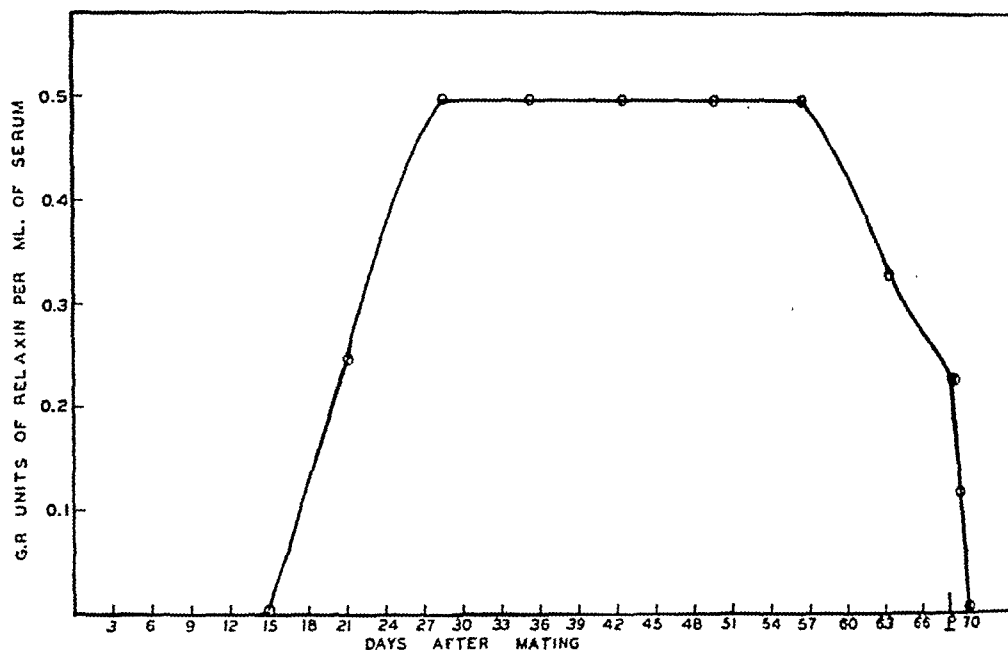
FIG. 1.

The relaxin content of the blood serum of pregnant and postpartum guinea pigs. Parturition (P) occurred 68 days after mating.

relaxin content of 5 G.P. units per gram. On the 63rd day of pregnancy the uterus still contained 10 G.P. units of relaxin per gram of fresh tissue while the placenta had only 2.5 G.P. units per gram.

Discussion. A series of 20 guinea pigs were palpated daily starting with the first day of pregnancy in order to determine the time at which relaxation of the symphysis pubis normally occurred. Relaxation was first detected on about the 20th day (range, 18 to 25 days), which compared favorably with the time at which relaxin first appears in the blood of the pregnant animals (Fig. 1.) It should be further pointed out that these two events may also be correlated with the time during which the placenta undergoes its most rapid development (Marshall[9]). There is a similar correlation in the rabbit between the development of the placenta and the appearance of relaxin in the blood (Marder and Money[6]). The maximum concentration of relaxin found in the blood of the pregnant guinea pig was 0.5 G.P. units per ml of serum which is one-twentieth the concentration

9 Marshall, F. H. A., 1922, The Physiology of Reproduction. Longmans, Green and Co., London.

found in the pregnant rabbit.

The fall in the relaxin content of the blood serum of the guinea pig previous to parturition is significantly different from the situation in the pregnant rabbit. In the rabbit the relaxin concentration is maintained at a plateau until parturition, at which time a precipitous drop occurs (Marder and Money[6]). However, there is some evidence which indicates that the situation in the pregnant woman may be similar to that in the guinea pig. It has been found that the amount of relaxin in the blood serum of pregnant women *is considerably greater during the first half of pregnancy than during the 8th month* (Pommerenke,[4] Abramson, Hurwitt, and Lesnick[5]). Thus, it would appear that in both the guinea pig and woman, unlike the rabbit, changes take place prior to parturition resulting in a decreased production of relaxin.

From the results obtained on the relaxin content of the uterus and placenta of the guinea pig at 56 and 63 days of pregnancy it would appear that the drop in the relaxin concentration of the blood serum may be due to a decreased output by the placenta. However it is not possible to account for this

with certainty and the problem is further complicated by the fact that a species difference may exist with regard to the part of the female reproductive tract that may serve as a source for relaxin.

Summary. Relaxin is found in the blood of guinea pigs on about the 21st day of pregnancy, at which time relaxation of the symphysis pubis may first be detected by palpation. The concentration of relaxin reaches a peak of 0.5 G.P. units per ml of blood serum on the 28th day and is maintained until the 63rd day when the concentration falls to 0.33 G.P. units per ml. Immediately after parturition a precipitous decline of the relaxin content of the blood serum takes place. The urine shows a concentration of 0.5 G.P. units of relaxin per ml at 42 and 56 days of pregnancy with a drop to 0.25 G.P. units by the 63rd day. The uterus contains about 10 G.P. units per gram of tissue on the 56th and 63rd day of pregnancy whereas the placenta shows 5 G.P. units per gram of tissue on the 56th day and 2.5 G.P. units on the 63rd day. It seems probable that the drop in serum relaxin in the latter part of pregnancy may be due to a decreased output from the placenta.

Enhancement of Effect of Chorionic Gonadotrophin on Ovarian Hyperemia in the Rat by Pituitary Extract.

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The earliest demonstrable effect of chorionic gonadotrophin on the ovary of rats is the appearance of hyperemia.[1] This reaction has been used with varying degrees of success for the rapid diagnosis of pregnancy. Attempts to enhance the effect of chorionic gonadotrophin on ovarian hyperemia led to our present use of a pituitary extract for this purpose. This was based on the well-known synergistic effect of certain anterior pituitary extracts in augmenting the ovarian response when used in conjunction with chorionic gonadotrophin.[2] The effective component of such pituitary extracts is not known. The earlier claims[3] that such "synergists" represent a distinct gonadotrophin no longer seems likely in view of later work[4] indicating that similar effects can be obtained with follicle-stimulating gonadotrophins (F.S.H.). The pituitary synergist† employed in these studies is reported to be chiefly F.S.H., with traces of luteinizing principle.[5] Prolactin was not demonstrable.

Methods and Results. The 28- to 32-day-old, 50 to 60 g immature albino female rats used were of a mixed strain of unknown identity. Chorionic gonadotrophin, the pitui-

2 a. Evans, H. M., Meyer, K., and Simpson, M. E., PROC. SOC. EXP. BIOL. AND MED., 1931, **28**, 845.

b. Evans, H. M., Meyer, K., and Simpson, M. E., *Am. J. Physiol.*, 1932, **100**, 141.

c. Fevold, H. L., Hisaw, F. L., Hellbaum, A. A., and Hertz, R., *Am. J. Physiol.*, 1933, **104**, 710.

d. Fevold, H. L., Hisaw, F. L., and Hertz, R., PROC. SOC. EXP. BIOL. AND MED., 1933, **30**, 914.

e. Leonard, S. L., PROC. SOC. EXP. BIOL. AND MED., 1932, **30**, 403.

f. Leonard, S. L., and Smith, P. E., *Am. J. Physiol.*, 1934, **108**, 22.

g. Smith, P. E., and Leonard, S. L., PROC. SOC. EXP. BIOL. AND MED., 1933, **30**, 1248.

h. Collip, J. B., Selye, H., Anderson, E. M., Thomson, D. L., *J.A.M.A.*, 1933, **101**, 1553.

5 a. Evans, H. M., Simpson, M. E., and Austin, P. R., *J. Exp. Med.*, 1933, **57**, 897.

b. Evans, H. M., Simpson, M. E., and Austin, P. R., *J. Exp. Med.*, 1933, **58**, 545.

c. Evans, H. M., Pencharz, R. I., and Simpson, M. E., *Endocrinology*, 1934, **18**, 601, 607.

* Schering Fellow in Gynecologic and Obstetric Endocrinology.

1 Zondek, B., and Sulman, F., *Vitamins and Hormones*, 1945, vol. 3, p. 328, Academic Press, N. Y.

TABLE I.
Hyperemia Response with Varying Dosages of Chorionic Gonadotrophin and Synergist.

Reaction time	Dose Chorionic Gonad. (I.U.)	Dose of synergist (units) and response										
		None Pos.	Neg.	0.044 Pos.	Neg.	0.022 Pos.	Neg.	0.011 Pos.	Neg.	0.0044 Pos.	Neg.	0.0022 Pos.	Neg.
2 hr	None	—	—	0	18	0	9	0	5	0	5	0	4
	0.60	19	6	4	0	8	0	5	0	5	0	4	2
	0.55	4	9	4	0	3	0	1	3	1	3	—	—
	0.50	0	11	5	0	3	0	0	3	—	—	—	—
	0.45	0	5	5	0	3	0	0	4	—	—	—	—
	0.40	0	5	3	0	3	0	1	2	—	—	—	—
	0.30	0	4	3	0	3	0	0	5	—	—	—	—
	0.20	0	4	0	3	0	3	—	—	—	—	—	—
	0.10	0	4	0	3	0	3	—	—	—	—	—	—
1 hr 15 min	None	—	—	0	4	—	—	—	—	—	—	—	—
	0.65	5	3	6	0	—	—	—	—	—	—	—	—
	0.60	4	5	5	0	—	—	—	—	—	—	—	—
1 hr	None	—	—	0	4	—	—	—	—	—	—	—	—
	0.70	1	7	3	0	—	—	—	—	—	—	—	—

tary synergist, or their combination was injected intraperitoneally into a total of 307 rats. The rats were asphyxiated with ether and autopsied after one, 1¼ or 2 hours. The reaction was considered positive if one or both ovaries were light to dark crimson, and negative if both were pale or pinkish. The details of the technic used have been described by Kupperman and Greenblatt.[6]

The results are given in Table I. There were no positive reactions with the synergist alone. Chorionic gonadotrophin in a dosage of 0.6 I.U. produced positive reactions in 76% of 25 rats. The percentage of positive results could not be increased by raising the dosage higher. When 0.0044 or more units of synergist were added to this minimal effective amount[‡] of gonadotrophin, strong positive reactions were present in 100% of 22 rats. With subminimal doses of chorionic gonadotrophin alone, positive reactions began to appear only after the injection of 0.55 I.U. or more. Strong positive reactions were noted in all rats injected with 0.30 to 0.55 I.U. of chorionic gonadotrophin if 0.022 or more units of synergist were added. The combinations of 0.2 I.U. or less of chorionic gonadotrophin and the maximum amount of synergist used, 0.044 units, did not produce positive reactions.

At 1¼ hours the percentage of positive reactions with the subminimal effective amount of chorionic gonadotrophin (0.65 I.U.) was increased from 62 to 100 with the addition of 0.044 units of the synergist, while the percentage of positive reactions with 0.60 I.U. was raised from 44 to 100. It is notable that though the minimal dosage of chorionic gonadotrophin at one hour was 3.0 I.U., positive reactions with 0.70 I.U. were increased from 15 to 100% with the addition of 0.044 units of the pituitary synergist.

The enhancement of the rat ovary hyperemia effect by this pituitary extract has been used to advantage by us in testing urines for the presence of pregnancy.

4 a. Evans, H. M., Simpson, M. E., Tolksdorf, S., and Jensen, H., Endocrinology, 1939, 25, 529.

b. Jensen, H., Simpson, M. E., and Tolksdorf, S., Endocrinology, 1939, 25, 57.

† The synergist was prepared from sheep pituitaries. The unit was defined as the amount of synergist which in combination with 15 I.U. of chorionic gonadotrophin will cause a 5-fold increase in ovarian weight over that of the uninjected controls. Human chorionic gonadotrophin was present in an amount equivalent to about 0.25 I.U. per mg (22.2 units) of synergist. The synergist was kindly furnished by Dr. E. Schwenk of the Schering Corp., Bloomfield, N.J.

5 Cutuly, E., Endocrinology, 1942, 31, 13.

6 Kupperman, H. S., and Greenblatt, R. B., South. Med. J., 1946, 39, 158.

‡ That amount which produced positive ovary hyperemia reactions in approximately 75% of 5 or more rats.

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The Activity of Streptomycin in the Presence of Serum and Whole Blood.[*]

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(Introduced by P. H. Long.)

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Previous studies[1] have shown that streptomycin resistant variants of Staphylococcus, Pneumococcus and Streptococcus grow as well in a bactericidal test as their parent susceptible strains. It was presumed that, in the presence of streptomycin, the additive effect of fresh whole blood could be demonstrated on both the susceptible and resistant strains. The effect on the latter, it was felt, would be minimal, but such investigation was of importance because of the numerous examples of strep- tomycin resistance which had been observed to develop during the course of therapy. No studies have been reported in which the susceptibility of the resistant organism was tested by the bactericidal technique in the presence of added streptomycin—a condition which would naturally occur in a patient under therapy.

The following studies were made to investigate the inter-relationships which may be present when bactericidal tests are performed with a strain of *Staphylococcus aureus* in the presence of streptomycin. In addition, the various important known components of the bactericidal test such as phagocytosis, hemolysis of red blood cells, immune serum and labile constituents were investigated. All

[1] Chandler, C. A., and Schoenbach, E. B., PROC. SOC. EXP. BIOL. AND MED., 1947, 64, 208.

these factors were controlled, wherever possible, by simultaneous duplicate observations in which the sensitive parent strain of the staphylococcus was used.

Materials and Methods. The strain used throughout the tests was a *Staphylococcus aureus* (Merck test strain SM) obtained from Sydenham Hospital. The strain of staphylococcus resistant to 1000 µg of streptomycin was derived from the parent strain as previously described.[1] Following initial experiments all cultures were transferred at intervals of 3 to 4 weeks on suitable culture media not containing streptomycin. No attempt was made to maintain the original virulence of the strains during the experimental period.

The susceptibility of the strains to streptomycin was determined by the use of a series of tubes of nutrient broth (PDW)† containing streptomycin in appropriate concentrations. Each tube, within the range, contained 10% less streptomycin as measured against the initial tube containing the most streptomycin in a regular progression, so that the differences from tube to tube were uniform. Each tube was inoculated with 0.05 cc of an 18-hour broth culture in such dilution as to yield an inoculum of about 200,000 organisms per one cc of medium. The tubes were incubated at 37°C and readings were made at the end of 24 hours.

The bactericidal power of freshly defibrinated human blood on the staphylococcus was determined in the following manner. Five hundredths cc of each 10-fold serial dilution (10^{-1} to 10^{-6}) of an 18-hour broth culture was added to 0.25 cc of freshly defibrinated human blood in each of 6 sterile pyrex tubes. These tubes were sealed, placed in a rotating box and maintained at 37°C for 24 hours. After preliminary observation, incubation without rotation was continued for another 24 hours, after which the tubes were opened and the contents cultured on blood agar plates. In order to determine the number of organisms added to each tube of blood, count plates were made of the 10^{-5} and 10^{-6} dilutions. This technique was varied as described in each group of experiments by the sub-

stitution of serum as a substrate and by the addition of immune serum, streptomycin, etc.

Phagocytic activity was measured in the following manner: 0.25 cc of whole, defibrinated blood was measured into sterile pyrex tubes. To the above tubes, 0.1 cc serum, either normal or immune was added. Streptomycin, in a final concentration of 500 µg was then introduced into some of the tubes. Fluid volumes in all tubes were made equal by the addition of appropriate amounts of broth. Finally each tube was inoculated with 0.05 cc of a 3-hour culture of either the susceptible or resistant strain. The tubes were then sealed in an oxygen flame, slowly rotated for 30 minutes at 37°C, broken open, and one drop of the contents of each was smeared on a slide as a blood film. The slides were then stained with Wright's stain and examined with the oil immersion lense. A count was made of the number of intracellular staphylococci contained in 25 polymorphonuclear leukocytes and the percentage of cells taking part in the phagocytosis was noted.

Growth curves were obtained by making bacterial counts at 2, 4, 6 and 24 hours. The number of organisms was estimated by making count plates of suitable dilutions of the culture. Estimation of turbidity in a photoelectric colorimeter with readings at hourly intervals was used in each instance to confirm these counts.

Immune rabbit serum was obtained in the following manner. A white albino rabbit was injected intravenously with a washed, heat-killed suspension of organisms. The suspension was prepared from an 18-hour broth culture of the susceptible parent strain of the staphylococcus. The rabbit received injections 4 times a week for 3 weeks. During the final week living organisms were used. The final agglutinative titre of the serum prepared as described above was 1-20 against both the susceptible strain and the resistant strain. Thread agglutination was observed when both these strains were grown in media containing this immune serum.

One lot (Winthrop-Pfizer Lot No. P4713) of streptomycin was employed throughout this study. It had a labeled potency of 1000

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† Peptone-Dextrose-Water.

TABLE I.
Bactericidal Studies.

Strain	Substrate	Minimum No. of organisms required to initiate growth per cc of blood or serum				
		Streptomycin, 0 μg	Streptomycin, 5 μg × million	Streptomycin, 10 μg × million	Normal rabbit serum	Immune rabbit serum
Parent susceptible	Whole blood Serum	420 520*	4.2 52.0*	42 52*	640 —	640 —

* Human serum.

TABLE II.
Proportion of Organisms in Resistant Staphylococcus Culture Growing at Indicated Streptomycin Concentrations.

Concentration of streptomycin in medium (μg per cc)							
0 colonies		500 colonies		750 colonies		1000 colonies	
No.	%	No.	%	No.	%	No.	%
700	100	700	100	600	85.7	600	85.7

units per milligram of pure base.

Results. (1) The sensitivity to streptomycin of the susceptible strain was re-evaluated in the light of previous reports that serum or whole blood inhibited the action of streptomycin.[2,3,4] In broth, the bacteriostatic range of streptomycin for the susceptible strain was 5 to 12 micrograms on repeated determinations. In the presence of 60% fresh human or rabbit serum (final concentration), this same strain was inhibited by 5 μg of the antibiotic when the inocula varied from 520 organisms to 5.2 million per cc. Growth occurred in the presence of 5 and 10 μg of streptomycin when a large inoculum was used (52,000,000 organisms).

In fresh whole human blood, inhibition occurred in a concentration of 5 μg with inocula ranging from 420 to 4.2 million. With 10 μg of streptomycin present, an inoculum of 42 million organisms was necessary to produce growth. In both the serum and whole blood assays, these findings were confirmed by sub-culture after 48 hours. This was deemed necessary to rule out erroneous inter-

pretations which may arise because of non-specific turbidity or hemolysis.

In summary, as shown in Table I, the susceptibility of this strain of staphylococcus to streptomycin was not affected by the presence of serum or whole blood within the limits of measurement which were employed. In addition, there was no real indication that the bacteriostasis achieved with streptomycin was augmented by the bactericidal activity of whole blood. In the absence of streptomycin, there was no indication that this strain of staphylococcus was affected by normal whole blood or when immune serum was added to the system.

(2) The resistant strain of staphylococcus had been derived in this laboratory from the parent strain and had been found to grow readily in broth containing more than 1000 μg of streptomycin. Colony counts performed by seeding agar plates containing either no streptomycin or up to 1000 μg of streptomycin revealed no essential difference in the number or type of colonies. This is shown in Table II. In the bactericidal test, as shown in Table III, the resistant strain was not inhibited by normal human blood or when immune serum was added to the system. Thus in all respects, except for its resistance to streptomycin, it resembled the parent strain.

When the resistant strain was grown in the

2 May, J. R., Voureka, A. E., and Fleming, A., *Brit. Med. J.*, 1947, **1**, 627.

3 Henry, R. J., Berkman, S., and Housewright, R. D., *J. Pharm. and Exp. Therap.*, 1947, **89**, 42.

4 Hobby, G. L., and Lenert, T. F., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 242.

TABLE III.
Bactericidal Studies.

Minimum No. of organisms required to initiate growth per cc of blood or serum

Strain	Substrate	Streptomycin, 0 µg	Streptomycin, 125 µg	Streptomycin, 500–1000 µg	Normal rabbit serum	Immune rabbit serum	Immune rabbit serum + 125 µg*	Immune rabbit serum + 500–1000 µg*
Resistant	Whole blood	320	320	320	340	340	340	340
	Serum	360†	360†	360†	280‡	280‡	280‡	280‡

* Streptomycin.
† Human serum.
‡ Rabbit serum.

TABLE IV.
Bactericidal Studies.

Minimum No. of organisms required to initiate growth per cc of blood or serum

Strain	Duration of growth, hr	Substrate	Normal rabbit serum	Normal rabbit serum + 125 µg*	Normal rabbit serum + 500 µg*	Immune rabbit serum	Immune rabbit serum + 125 µg*	Immune rabbit serum + 500 µg*
Resistant	24	Whole blood	3600	3,600,000	360,000	360	360,000	3,600,000
	48	,,	340	—	—	340	340	340
Resistant	24	Rabbit serum	280	2800	280,000	280	280	28,000
	48	,,	280	280	2800	280	280	280

* Streptomycin.

TABLE V.
Bactericidal Studies.

Minimum No. of organisms required to initiate growth per cc of serum

Strain	Time inoculated after mixing	Substrate	Normal rabbit serum	Normal rabbit serum + 125 µg*	Normal rabbit serum + 500 µg*	Immune rabbit serum	Immune rabbit serum + 125 µg*	Immune rabbit serum + 500 µg*
Resistant	0	Rabbit serum	280	280	2800	280	280	280
	24 hr	Rabbit serum	160	1600	1600	160	160	16,000

* Streptomycin.

TABLE VI.
Phagocytic Studies.

Strain	Normal rabbit serum		Immune rabbit serum		500 μg streptomycin		Normal rabbit serum + 500 μg streptomycin		Immune rabbit serum + 500 μg streptomycin	
	No. of cocci phagocyted	Cells with cocci	No. of cocci phagocyted	Cells with cocci	No. of cocci phagocyted	Cells with cocci	No. of cocci phagocyted	Cells with cocci	No. of cocci phagocyted	Cells with cocci
Parent susceptible	204	68%	625	94%	126	40%				
Resistant	960	92%	1100	92%	832	72%	997	84%	775	72%

presence of 60% rabbit serum containing streptomycin, definite inhibition of growth was observed. With as little as 125 μg of streptomycin per cc, this strain, resistant to 1000 μg per cc, showed retardation in growth after 24 hours when small inocula (200 to 300,000 organisms per cc) were used. At the end of 48 hours, however, growth equal to that in the controls was observed. A similar pattern of growth inhibition with streptomycin was noted in the media containing whole blood with or without added immune serum. Inhibition was somewhat more apparent in these latter experiments because hemolysis was delayed. These results are summarized in Table IV.

An investigation was then undertaken into the cause of this transient inhibition of growth. That streptomycin might be inactivated or bound by one of the serum constituents, was considered a distinct possibility. If so, this would account for the subsequent escape from the inhibitive effects of streptomycin. Accordingly, duplicate experiments were devised in which normal rabbit serum which had been inactivated by heat, with and without added immune rabbit serum, and to which streptomycin had been added in concentrations of 125 and 500 μg per cc was inoculated with organisms at 2 different times. One set was inoculated immediately and incubated for 24 hours while the duplicate set was incubated for 24 hours after which it was inoculated with organisms and again incubated for 24 hours. As shown in Table V, no difference was observed between the set inoculated immediately and that inoculated after 24 hours and, therefore, the escape from inhibitive effects of streptomycin after 24 hours could not be attributed to inactivation of streptomycin.

The lag observed when the resistant strain was grown in the presence of streptomycin cannot be attributed to the non-homogeneous distribution of streptomycin sensitive organisms as suggested by Berkman et al.[5] As shown in Table II, almost all organisms were able to grow at higher streptomycin levels

5 Berkman, S., Henry, R. J., and Riley, D. H., J. Bact., 1947, 53, 567.
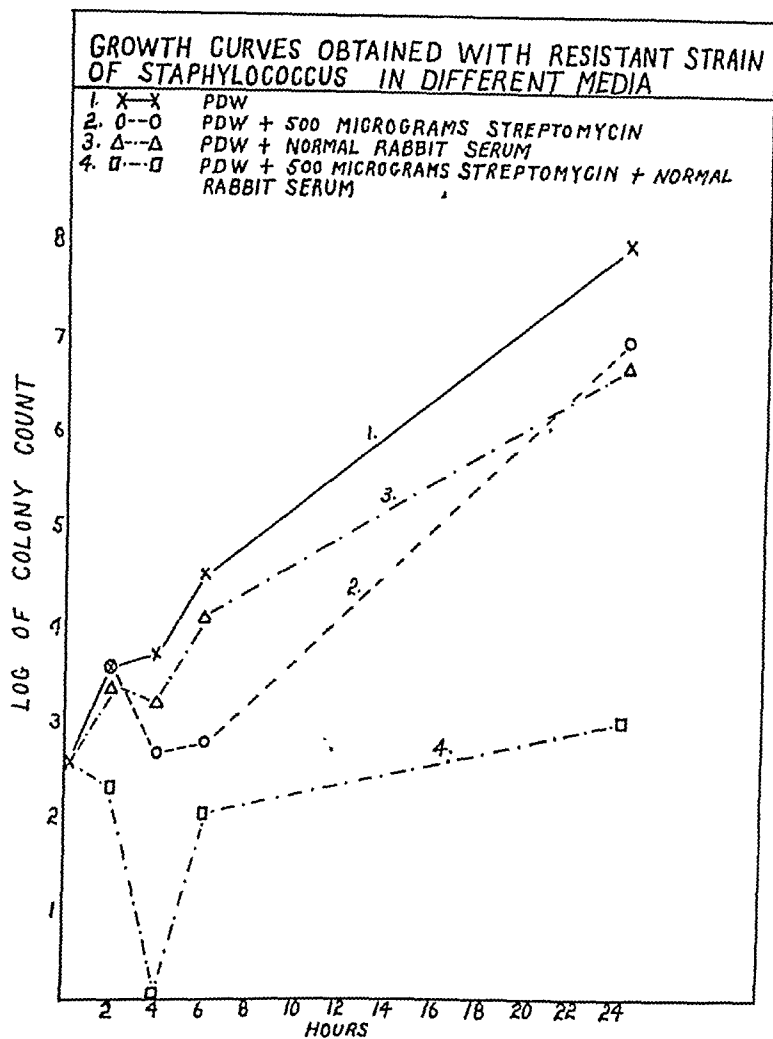
FIG. 1.

than were necessary to demonstrate this lag.

These observations are not necessarily in conflict with Price *et al.*[6] and Berkman and his co-workers[5] who found a marked difference in sensitivity to streptomycin when assayed in a somewhat deficient as compared with a highly nutritive medium. It would appear, however, from our observations that beyond a certain point progressive enrichment, although promoting growth of organisms, does not affect the final streptomycin titre.

Another possibility which was entertained was that streptomycin had stimulated phagocytosis and that, with the death of the leukocytes after 24 hours, growth had reappeared.

As shown in Table VI, it was found that in the presence of streptomycin, the phagocytic activity of the white blood cells was depressed in every instance and, therefore, the initial bacteriostasis could not be explained on the basis of enhanced phagocytosis at these streptomycin levels.

In the light of the above results, it seems probable that the only explanation of the in-

6 Price, C. W., Randall, W. A., Chandler, V. L., and Reedy, R. J., *J. Bact.*, 1947, **53**, 481.

hibition of growth in the first 24-hour period in the presence of streptomycin, was a retardation in the rate of growth. Despite the observation that growth of the resistant strain in the absence of streptomycin did not differ materially from the susceptible strain in media containing broth or serum, this strain of staphylococcus, resistant to more than 1000 μg of streptomycin per cc, was markedly inhibited in its rate of growth by much lower concentrations of the antibiotic in the first 24-hour period. This inhibitory activity could be demonstrated with as little as 125 μg of streptomycin per cc. It was augmented when serum was present in the medium. Sample growth curves in streptomycin and non-streptomycin containing media are presented in Fig. 1. This type of growth curve resembles that noted with the sulfonamides which is apparent in the early phase of growth when small inocula are used.

Discussion. With the susceptible parent strain of staphylococcus, the addition of fresh whole blood to streptomycin did not augment streptomycin activity. On the other hand, the efficiency of the streptomycin was apparently not affected by the presence of blood and its constituents. It was, therefore, difficult to believe that the streptomycin was bound to some protein constituent in the serum unless the resultant combination retained the antibacterial properties of the antibiotic.

Inhibition of the rate of growth of a highly resistant strain in the presence of streptomycin was observed during the first 24 hours of incubation when the concentration of the latter was far below 1000 μg per cc. An explanation of this observation may be that the nutritional requirements of the organism had been modified with the acquisition of streptomycin resistance. The resistant strain appeared to grow as readily as the parent susceptible strain in the usual nutrient media, in the presence of serum and even in fresh whole human blood. When streptomycin was present in these media, however, the delay in the rate of growth of the resistant strain seemed to indicate that some nutritional factor or necessary enzymatic process had been materially affected. Eventually the resistant organ-

ism escaped from this streptomycin inhibition. The nature of this reaction may be of prime importance for the elucidation of the mode of action of streptomycin on microorganisms and possibly also the nature of the escape mechanism associated with resistance. These investigations are being continued at present.

These data may indicate that under normal conditions 2 alternative growth mechanisms are available to the normal organism. A general discussion of such mechanisms has been reviewed by Dubos.[7] In this study it would appear that one metabolic or synthetic pathway is blocked by streptomycin and another is insufficiently developed to permit normal growth. With the development of resistance, the second mechanism is augmented but apparently is still insufficient for optimal growth when streptomycin is blocking the first mechanism.

It is possible that the inhibitory activity of streptomycin is related to its combination with desoxyribonucleic acid in the bacterial cell.[8] Nucleoprotein metabolism is important in the growth and reproduction of bacteria. The combination of nucleic acid with streptomycin may interfere with the synthesis or utilization of nucleoproteins. Studies on the mode of action of penicillin have demonstrated that with *Staphylococcus aureus* dissimilation of ribonucleic acid was inhibited without any effect upon glucose oxidation.[9] The reaction inhibited by penicillin is probably the dissimilation of a pentose.[10] It has been claimed that streptomycin would also exhibit an inhibitory effect on the same reaction.[10]

When a strain resistant to streptomycin is isolated from a patient, it may be inferred that despite its ability to grow in high concentrations of the antibiotic *in vitro*, its rate of

[7] Dubos, R. J., *The Bacterial Cell*, 1945, Harvard University Press, Chapter VIII, Bacteriostatic and Bactericidal Agents, 275.

[8] Cohen, S. S., *J. Biol. Chem.*, 1947, **168**, 511.

[9] Krampitz, L. O., and Werkman, C. H., *Arch. Biochem.*, 1947, **12**, 57.

[10] Krampitz, L. O., Green, M. N., and Werkman, C. H., *J. Bact.*, 1947, **53**, 378.

growth in the body may be retarded by even small concentrations of the antibiotic. One should expect, therefore, that such resistant strains would have difficulty in maintaining themselves due to phagocytic mechanisms available *in vivo*. The impairment of phagocytosis by streptomycin should be studied in greater detail so that this important cellular factor in immunity may be more clearly defined.

Summary 1. The activity of streptomycin on a susceptible strain of *Staphylococcus aureus* was not changed in the presence of serum or whole blood. 2. The addition of fresh whole blood with or without immune serum did not augment streptomycin activity. 3. A resistant strain of *Staphylococcus aureus* was inhibited during the first 24 hours by streptomycin in low concentrations. This effect was augmented when serum was added to the medium. 4. Direct growth curves indicate that this inhibition by streptomycin of a resistant strain was attributable to interference with growth of the organism rather than inactivation of labile constituents. 5. Phagocytosis in the presence of high concentrations of streptomycin appears to be impaired. 6. The bacteriostatic activity of streptomycin was manifest on both susceptible and resistant strains. With the latter, this inhibition was transitory. The bacteriostatic mechanism and bactericidal mechanism are dissociated phenomena.

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Culture Procedures in Microbiologic Assays with *L. arabinosus* and *L. casei.*

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In the application of microbiological assay procedures, there have arisen questions relative to possible differences in the response of the test organisms as a result of variations in handling the stock cultures. Several investigators have mentioned the possible influence of stock culture media and conditions to which it is subjected on the subsequent response of an organism under assay environment, but few have made studies to determine the nature and extent of these influences. The most detailed work which has come to our attention has been the culture studies on *L. arabinosus* and *L. casei* by Nymon and Gortner.[1]

The present report is concerned with some notes made on frequency of transfer of stock cultures, preparation and dilution of inoculum and temperature and time of incubation of the assay tubes as these factors affect the assay.

Observations were made for a period of 6 months on the effect of frequency of transferring stock cultures of *L. arabinosus* on the subsequent growth response of this organism to graded amounts of nicotinic acid in the U.S.P.[2] assay medium. From a stab culture of *L. arabinosus* carried in agar medium (agar 1.5%, dextrose 1% and yeast 1%) 5 transfers were initially made into fresh agar medium of the same composition. After incubation at 30°C for 24 hours these cultures were stored in a refrigerator. One, designated "original", was maintained in the original stab without further transfer. The others were transferred at intervals of one, 2, 3, and 4 weeks respectively throughout the experiment. After incubation of the transplant for 24 hours it was returned to the refrigerator

TABLE I.
Effect of Frequency of Transfer of *L. arabinosus* on Titration Values in Nicotinic Acid Determinations After 24 Weeks.

	Titration values in cc 0.1N NaOH				
Micrograms standard nicotinic acid	0.1	0.2	0.3	0.4	0.5
Original culture	3.3	4.8	6.4	7.4	8.3
Culture transferred weekly	3.3	5.1	6.9	8.4	9.0
,, ,, 2-week intervals	3.3	5.1	7.1	8.2	8.7
,, ,, 3- ,, ,,	3.4	5.2	7.2	8.3	8.6
,, ,, 4- ,, ,,	3.4	5.1	7.1	7.9	8.6

until the next transfer period. Periodic comparisons of microbiological standard response curves obtained with each of these cultures showed that there was no difference after 20 weeks. Only at the end of 24 weeks was there evidence that the "original" culture gave titration values differing from those obtained with the other 4 cultures. Results are given in Table I. Infrequent transfer over a longer period might eventually produce adverse effects, but these experiments indicate a remarkable stability of the *L. arabinosus* culture used.

The results of experiments to determine whether differences in incubation temperatures of the assay tubes caused any change in

TABLE II.
Effect of Temperature and Time of Incubation of *L. arabinosus* on the Titration Values in Nicotinic Acid Determinations.

Exp. No.	Temp. of incubation, °C	Time of incubation, days	Titration	
			Blank	Maximum
			cc 0.1N NaOH	
1.	34	3	1.4	9.6
	35	3	1.4	9.7
2.	34	1	0.6	5.7
	37	1	0.6	5.7
	34	2	0.7	8.3
	37	2	0.7	8.3
	34	3	0.8	9.1
	37	3	0.8	8.4
3.	30	1	1.0	6.1
	33	1	1.1	6.1
	30	2	1.2	8.3
	33	2	1.2	8.2
	30	3	1.3	9.3
	33	3	1.2	9.3
	30	4	1.2	10.4
	33	4	1.3	10.2
	30	5	1.2	11.2
	33	5	1.3	11.4
	30	6	1.3	11.1
	33	6	1.4	11.1

the behavior of *L. arabinosus* are presented in Table II. Two series of tubes incubated at 34°C and 35°C respectively showed identical titration values after 3 days. Two series of assay tubes incubated for periods of one, 2, and 3 days at 34°C and 37°C respectively, gave practically the same titration values after the first and after the second day but gave slightly higher values in the 34°C series after 3 days. In a third experiment incubation temperatures were held at 30°C and 33°C for periods of one to 6 days inclusively with no significant differences observed between any of the paired members. Titration values increased with increased period of incubation reaching a peak on the fifth day and remaining the same through the sixth. Duplicate titrations, the averages of which are given in the table, were more uniform after 3 days incubation than at the end of a shorter period.

Inoculum is usually prepared from a broth culture after incubation for 20-24 hours in the basal medium used in the assay. It has been found, in the case of *L. arabinosus*, that subsequent storage of the incubated culture in a refrigerator for an additional 24 hours before preparation of the inoculum has no effect upon the assay results.

L. casei is known to be more susceptible to environmental influences than *L. arabinosus*. In Table III are data showing the effects of age and concentration of inocula of *L. casei* on the response of this organism to graded amounts of folic acid. A modification of the U.S.P. niacin medium[2] was used for these comparisons. Inocula were prepared from broth cultures incubated at 30°C for 16, 24, 40, and 48 hours. In addition there were included 16 hour broth cultures to which had been added a preparation containing strep-

TABLE III.

Effect of Age of Culture and Concentration of Inoculum on Titration values in Folic Acid Determination Using *L. casei*.

Micrograms standard folic acid	0	.0002	.0004	.0006	.0008	.001
			cc 0.1N NaOH			
16 hr culture—undil.	3.4	5.5	6.9	8.3	9.3	10.5
16 ,, ,, (containing strepogenin)—undil.	3.1	5.0	6.5	7.9	9.1	10.4
16 ,, ,, ,, ,, ,,	3.1	5.1	6.5	7.8	9.1	10.2
24 ,, ,, undil.	3.3	5.2	6.7	8.1	9.4	10.5
24 ,, ,, dil.	3.1	5.1	6.5	7.8	8.8	10.1
40 ,, ,, undil.	1.2	1.3	1.4	1.6	1.8	1.85
40 ,, ,, dil.	2.8	4.5	5.9	6.9	7.8	8.7
48 ,, ,, undil.	1.4	1.9	2.4	2.9	3.2	3.5
48 ,, ,, dil.	2.4	4.2	5.2	6.2	7.0	7.8

ogenin that was found to be active in accelerating the initial growth of *L. casei*, on purified media as originally reported by Sprince and Woolley.[3] An undiluted portion of each inoculum was tested as well as a portion diluted to the same turbidity as the 16 hour preparation without the strepogenin. There was little difference in the response to graded amounts of folic acid shown by inocula prepared from the 16 hour culture, with or without strepogenin, and the 24 hour culture. Dilution of the inoculum from either the 16 hour culture with strepogenin or the 24 hour culture did not alter the degree of this response. The concentrated inocula from the 40 and 48 hour cultures gave growth in all of the tubes insignificantly above that in the blanks. The diluted inocula for these 40 and 48 hour cultures gave a usable growth range but one that was considerably less than that produced by inocula from the younger cultures. It appears that the best results with *L. casei* can be obtained by preparing inocula from cultures not more than 24 hours old.

Discussion. Failure of the frequency of transfer of our stock culture of *L. arabinosus* to affect the nicotinic acid assay results does not agree with the findings of Nymon and Gortner. These investigators reported a gradually decreased linearity of response in their culture of *L. arabinosus* transferred every 3 or 4 weeks in yeast extract-glucose-agar medium with incubation at 37°C. This response was improved by culturing in enriched media, reducing the incubation temperature to 30°C,

the optimum reported by Bergey *et al.*[4] for *L. arabinosus*, and increasing the frequency of transfer. They did not include a consideration of incubation temperatures of the assay tubes in their report.

Price and Graves[5] found, in the determination of riboflavin by *L. casei*, that incubation temperature variations of 4-5°C from the optimum of 40-42° for this organism resulted in a decrease of 25% in the titration values. In the present study, no such striking differences in the behavior of *L. arabinosus* were observed with temperature variations in the incubation of assay tubes in the nicotinic acid determination. However, it is well recognized that *L. casei* is more readily affected by environmental conditions than *L. arabinosus*. Following earlier recommendations of Snell and Strong[6] for the preparation of inocula, Bird *et al.*[7] obtained more satisfactory results when they used diluted inocula. This agrees with our findings for inocula made from older cultures of *L. casei*. On the other hand no difference could be seen in undiluted and diluted inocula from cultures not more than 24 hours old. Inocula from young cultures were superior to inocula from old cultures even though the latter had been diluted.

[4] Bergey, D. H., Breed, R. S., Murray, E. G. D., and Hitchens, A. P., *Bergey's Manual of Determinative Bacteriology*, 1939, Baltimore, 5th edition.

[5] Price, S. A., and Graves, H. C. H., *Nature*, 1944, 153, 461.

[6] Snell, E. E., and Strong, F. M., *Ind. and Eng. Chem., Anal. Ed.*, 1939, 11, 346.

[7] Bird, O. D., Bressler, B., Brown, R. A., Campbell, C. J., and Emmett, A. D., *J. B. C.*, 1945, 159, 631.

[3] Sprince, H., and Woolley, D. W., *J. Exp. Med.*, 1944, 80, 213.

Summary. The results indicate that the culture of *L. arabinosus* used in these studies for microbiological assay was not easily affected by frequency of transfer of stock cultures or by incubation of assay tubes at fixed temperatures between 30°C and 37°C. In the case of the culture of *L. casei*, both the dilution of the inoculum and the age of the culture from which it was prepared affected assay titration values.

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Recovery of Virus from Throats of Poliomyelitis Patients.*

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In discussions of the epidemiology of poliomyelitis much importance has been placed on the presence or absence of virus in the nose and throat of patients and of associates in so far as such findings might support theories of transmission of the disease by means of droplets. A review of literature on the subject in 1938[1] indicated that virus was reported in the nasopharynx, tonsils or trachea of patients in 15% of 105 examinations made during the first 5 days of illness and in 7% of 182 made later. Conflicting evidence has been presented during the last decade. Sabin and Ward found virus in the pharyngeal wall of 4 of 7 patients dead within 6 days of onset,[2] and Kessel *et al.* reported positive results with tonsil-adenoid tissue in 3 of 6 autopsy specimens.[3] Howe and Bodian detected virus in pooled throat swabs from at least 2 of 28 healthy children and from 1 of 6 juvenile, familial associates of cases; the positive associate may have been a nonparalytic case since the child had fever and diarrhea at the time of collection of the specimen.[4] Oropharyngeal swabs obtained by the same authors, within 3 days after onset of illness from 10 of 23 patients yielded virus, but none of 13 swabs taken between the 4th and 9th days was positive.[5] In a similar study by Horstmann, Melnick and Wenner, only one of 19 swabs and one of 15 washings obtained during the first week of illness contained virus;[6] the specimens from 10 of the 19 patients in the study were obtained within 3 days after onset of symptoms. Kramer, Hoskwith and Grossman[7] tested 18 washings of the nasopharynx and recovered virus from 2 which had been collected 5 and 9 days after onset, respectively. Ward and Walters tested material from the nose and throat of 19 patients between the second and fourth days of illness. Virus was isolated from cloth masks over the nose and mouth of two patients, from a nasal swab of one of these, from pharyngeal swabs of the second and from 5 others.[8]

This report describes attempts to recover virus from pharyngeal washings of a group of poliomyelitis patients during the outbreak

[1] Vignec, A. J., Paul, J. R., and Trask, J. D., *Yale J. Biol. and Med.*, 1938, **11**, 15.

[2] Sabin, A. B., and Ward, R., *J. Exp. Med.*, 1941, **73**, 771.

[3] Kessel, J. F., Moore, F. J., Stimpert, F. D., and Fisk, R. T., *J. Exp. Med.*, 1941, **74**, 601.

[4] Howe, H. A., and Bodian, D., *Am. J. Hyg.*, 1947, **45**, 219.

[5] (a) Howe, H. A., Wenner, H. A., Bodian, D., and Maxcy, K. F., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 171; (b) Howe, H. A., Bodian, D., and Wenner, H. A., *Bull. Johns Hopkins Hosp.*, 1945, **76**, 19.

[6] Horstmann, D. M., Melnick, J. L., and Wenner, H. A., *J. Clin. Invest.*, 1946, **25**, 270.

[7] Kramer, S. D., Hoskwith, B., and Grossman, L. H., *J. Exp. Med.*, 1939, **69**, 49.

[8] Ward, R., and Walters, B., *Bull. Johns Hopkins Hosp.*, 1947, **80**, 98.

<div align="center">TABLE I.</div>

Dallas Throat Washings.

Day after onset of symptoms specimen was collected	3	4	6	7	7	7	8	9	9	11	11	11	11	13	13	13
Age of patient	6	4	11	3	11	14	4	3	13	3	4	11	15	5	13	14
Virus present	—	—	—	—	—	—	—	—	—	+	+	—	—	—	—	—

Detroit Throat Washings.

All 1 to 3

Day after onset																
Age	7	7*	12	13	15	15	15	16	18		3	4	5	6	7*	7	10
Virus present	(+	—	—	—	+	—	—	+	—)		(+	+	+	—	—	—	+)

Washing   Swab

* Washing and Swab combined.

in Dallas, Texas, 1943 and from swabs and washings from patients in Detroit, Michigan during 1944.

Materials and Methods. The washings from patients in Dallas were obtained by expressing 3 to 5 ml of saline into each nostril with a needleless syringe and collecting the fluid in a paper cup after it ran into the throat. The patient then gargled 20 to 30 ml of saline which also were collected in the cup. Washings in Detroit consisted of 20 ml of gargled, distilled water. Swabs were obtained by use of cotton on applicator sticks. These were rolled over the tonsillar areas and the posterior oropharynx then twirled in 1 to 2 ml of saline contained in a lusteroid vial and finally pressed against the side of the tube to express the saline. All specimens were stored under dry ice refrigeration until prepared for inoculation into rhesus monkeys.

Throat washings from Dallas were concentrated by centrifugation of one or two 7 ml samples per specimen in an air-driven centrifuge at 30,000 r.p.m. for one hour.[†] Supernatant fluid was removed, leaving 0.5-0.75 ml in the tube, and saved for intraperitoneal injection in a monkey that also received the sterile sediment intracerebrally. The sediment was resuspended in the fluid remaining by use of a glass rod; sediments from the companion tubes were combined when there were 2 samples. Bacteria were inactivated by addition of ether which was evaporated *in vacuo* after 1 to 7 days of contact in the refrigerator. In some specimens bacteria survived the treatment with ether and were inactivated by the addition of phenol to a final concentration of

0.5%. One to 1.5 ml of each specimen was injected intracerebrally into a rhesus monkey.

The throat swab fluids from Dallas and the throat washings from Detroit were treated with ether or ether and phenol and inoculated intracerebrally. The latter, however, were first concentrated to a volume of 1 to 2 ml by evaporation from a cellophane "Visking" casing placed before an electric fan.

Results. All monkeys were observed for a period of one month after injection. Animals that developed paralysis were considered positive for poliomyelitis if histological examination of the nervous system revealed characteristic pathology of the disease. The results of the tests are shown in Table I.

Virus was detected in 2 of 16 Dallas patients, one of the spinal and one of the bulbar type. Both specimens were collected on the eleventh day after onset of symptoms. Eleven other specimens obtained between the 3rd and 11th days were negative. Of the specimens collected in Detroit all within 3 days after onset, 3 of 9 throat washings contained virus as did 4 of 7 throat swabs. One of the 4 positive swabs was from a bulbar paralytic and one from a nonparalytic patient.

Discussion. The results obtained tend to support the impression that swabs are as good, if not a better method of recovering virus from the throat than throat washings even though, as in this study, the latter are concentrated before testing.

The evidence to date shows that poliomyelitis virus is present in the throats of patients and may be readily detected early in the illness. The present study duplicates the report of Howe, Bodian and Wenner[5-b] in demonstrating virus in 43% (7 of 16) of those

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[†] The centrifugation was kindly made by Dr. R. W. G. Wyckoff of this laboratory.

Fig. 1

ed as the chief routes of contamination from the patient. Likewise the more frequent recovery of poliomyelitis virus from the bowel than from the throat has in recent years tended to emphasize the former as the more important portal of exit.

Another argument favoring enteric over respiratory spread is the seasonal prevalence of the disease. As shown in the figure, poliomyelitis has a seasonal occurrence similar to typhoid, dysentery, typhus and malaria and unlike the disease spread by the respiratory route; diphtheria has a unique curve that perhaps may be explained as resulting in part from increased incidence associated with reopening of schools. The temptation exists to reason from analogy and to suggest that outbreaks of poliomyelitis like those of typhoid-dysentery are started by enteric carriers; the subsequent spread of infection may depend on either or both respiratory or enteric contamination of the environment. More data are needed particularly in regard to the relative importance of the case and of the asymptomatic carrier in spreading the illness and in regard to physiologic effects of nutrition and of environmental temperature on susceptibility to the disease. Meanwhile, until evidence to the contrary is obtained it would seem necessary to consider the throats of poliomyelitis patients as important potential sources of infection.

patients tested within 3 days of onset. However, the fact that virus was detected in two throat washings 11 days after onset suggests that its persistence may be quite variable.

The presence of an agent in the throat does not necessarily mean that the nose and mouth are the chief portals of exit for the germ. For example bacilli are reported present in the mouths of approximately 50% of typhoid patients[9] yet the stools and urine are regard-

Summary. Virus was recovered from 4 of 7 throat swabs and from 3 of 9 throat washings of poliomyelitis patients collected during the first 3 days after onset of symptoms. In another series of 16 throat washings collected from 3 to 13 days after onset, virus was isolated from 2 patients 11 days after onset of disease.

9 (a) Purjesz, B., and Perl, O., *Wien. Klin. Wchnschr.*, 1912, 25, 1494; (b) Gould, C. W., and Qualls, G. L., *J. A. M. A.*, 1912, 58, 542.

16137

Effects of Gonadotrophic Hormones on Lactation.*†

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The inhibiting effect of steroids on lactation is mediated through the ovaries[1,2,3] and is probably due to the hormonal secretions of the large corpora lutea formed under the influence of the steroids.[4] Since the ovaries are normally stimulated by gonadotrophins of pituitary or chorionic origin, we decided to investigate the effect of these hormones in lactating rats.

It has been reported that gonadotrophins from pregnancy urine inhibit lactation in the mouse and rat; the effective daily dose varies from 10 R.U. to 125 R.U.[5,6,7] Later studies showed, however, that more purified chorionic hormone possesses only a slight inhibitory effect, even when injected at the dose level of 100-500 R.U.;[8,9] its effectiveness can be increased by estrogens.[10] The report that pregnancy urine extracts were active in castrate animals[6] could not be confirmed.[9,11]

Pregnant mare serum has a marked inhibitory influence on lactation and results in the death of the young.[9]

Anterior pituitary extract, although active in adrenalectomized rats treated with cortin[12] has no effect in normal rats.[9] Inhibition of lactation has been obtained in combining such extracts with chorionic gonadotrophins.[13]

The present work was undertaken in order to study the effects of these hormones under the same conditions used previously for the steroids and to see whether some correlations could be established between the ovarian changes elicited by gonadotrophins and the degree of inhibition of lactation.

Forty-one albino rats weighing 250-300 g originating from the same colony and fed exclusively on purina fox chow were used. The number of young in the litters was reduced or brought up to 6 within 24-36 hours of parturition, time at which the treatment was initiated. The mothers were divided into 6 groups treated as follows: Group I, no treatment; Group II, lyophilized beef anterior pituitary preparation (L.A.P.) at the daily dose of 40 mg; Group III, chorionic gonadotrophin (A.P.L.) at the daily dose of 150 I.U.; Group IV, A.P.L. at the daily dose of 150 I.U. during the first 6 days, 300 I.U. from the 6th to the 13th day, and 450 I.U. from the 13th to the 16th day; Group V, pregnant mare serum (P.M.S.) at the dose of 300 I.U.; Group VI, P.M.S. at the dose of 600 I.U. All these preparations in a dry form were suspended or dissolved in a

1 Anselmino, K. J., and Hoffman, F., *Zentralbl. f. Gynak.*, 1936, **69**, 501.

2 Folley, S. J., and Kon, S. K., *Proc. Roy. Soc., London, B*, 1938, **124**, 476.

3 Barsantini, J. C., Masson, G., and Selye, H., *Rev. Canadienne Biol.*, 1946, **5**, 407.

4 Barsantini, J. C., and Masson, G., *Endocrinology*, 1947, **41**, 299.

5 Enzmann, E. V., and Pincus, G., *Am. J. Physiol.*, 1933, **103**, 30.

6 Jongh, S. E. de, *Acta brev. Neerland*, 1933, **3**, 88.

7 Connon, F. E., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 52.

8 Hathaway, I. L., Davis, H. P., Reece, R. P., and Bartlett, J. W., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 214.

9 Edelmann, A., and Gaunt, R., *Physiol. Zool.*, 1941, **14**, 373.

10 Reece, R. P., Bartlett, J. W., Hathaway, I. L., and Davis, H. P., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 183.

11 Jongh, S. E. de, and van der Woerd, L. A., *Acta brev. Neerland*, 1939, **9**, 26.

12 Gaunt, R., and Tobin, C. E., *Am. J. Physiol.*, 1936, **115**, 588.

13 Selye, H., Collip, J. B., and Thomson, D. L., *Endocrinol.*, 1934, **18**, 237.

TABLE I.
Average Figures on the Growth Rate of Litters and Percentage of Survivals Following Treatment of Mothers.

Groups	Treatment	No. of litters	Initial body wt	Avg body wt on day			% of litters surviving on day		
				5th	10th	16th	5th	10th	16th
1	0	12	6.8	10.6	17.3	26.2	96	96	92
2	L.A.P. 40 mg/day	7	7	12.5	17.5	24.3	97	97	97
3	A.P.L. 150 I.U./day	6	7	10.9	15.8	19.7	100	100	100
4	A.P.L. 150-450 I.U./day	6	7.7	11.7	18.4	21.8	100	100	100
5	P.M.S. 300 I.U./day	5	6.3	11.2	14.8	15.	92	92	18
6	P.M.S. 600 I.U./day	5	6.2	9.6	14.5	13.2	100	71	37

physiologic saline solution; the daily dose was administered in 2 subcutaneous injections. The criteria of efficiency of lactation were provided by the growth curve of sucklings and also by the number of deaths among the young. The mothers were sacrificed on the 17th day, or before, whenever all the young from the same litter died. At autopsy, organs were removed, placed in fixative fluid, then weighed and examined histologically.

L.A.P. had no influence on the growth or the mortality of the young, in spite of a certain degree of toxicity as evidenced by the local reactions near the points of injection. A.P.L. exhibited some inhibitory effect; although the mortality was nil in Groups III and IV, the growth was somewhat retarded, as can be seen from Table I. This effect on growth was more evident from the daily average weight of litters; it reached a maximum around the 14th day, then plateaued or even decreased. P.M.S., the most active preparation, influenced the growth and the mortality of young; the degree of inhibition, however, did not differ significantly whether a dose of 300 or 600 I.U. was administered.

On microscopic examination, only the mammary glands of Group II did not differ from those of Group I (controls). The lumina of ducts and acini were dilated and filled with homogeneous secretion; there was an almost complete disappearance of inter- and intra-lobular connective tissue.

Mammary glands of Groups V and VI presented a marked variation from the normal controls. There was a marked regression of the glandular tissue. The acini were fairly indistinct and their lumina were filled with a vacuolar material. The acinar cells, which

were cuboidal in type with large nuclei, showed no activity. The lobular masses were separated from each other by connective tissue. These glands were not secreting and presented signs of involution.

The picture of mammary glands of Groups III and IV was intermediary between that of controls and that of P.M.S.-treated rats. The acini were not distended by milk and the cells were low cuboidal in type but still secreting. The lobular masses were more distinct due to a decreased activity of the glandular tissue.

As expected, ovaries of all experimental groups were markedly stimulated. The ovarian weights for Groups II to VI were 138, 207, 257, 439 and 263 mg respectively to compare with 56 mg for the controls. On histologic examination the ovaries of Group II were found to contain numerous but small corpora lutea which gave to the organ a mulberry appearance. Those of Groups III to VI were more intensely luteinized; with P.M.S. the corpora lutea were larger than with A.P.L. and in many instances large vesicular or hemorrhagic follicles were observed. Since corpora lutea of the pregnancy type which are associated with inhibition of lactation can be identified by the size of the individual cells, we decided to draw a representative cell of the corpora lutea from each group. From Fig. 1 it can be seen that only the luteal cells of rats treated with P.M.S. presented an hypertrophy similar to that of normal pregnancy.

Although the experimental conditions of our studies and the criteria of activity were somewhat different from those used by others, the results are identical: P.M.S is the most active inhibitor of lactation, then comes
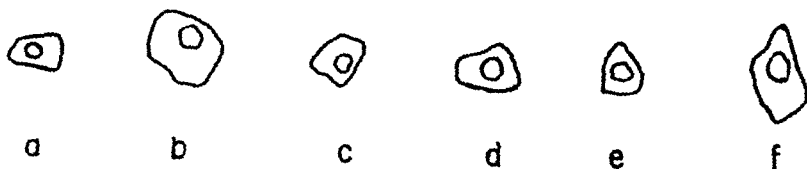
FIG. 1.
Camera lucida drawings of corpora lutea cells. (a) During lactation; (b) during pregnancy; (c) during lactation and A.P.L.; (d) during lactation and L.A.P.; (e) during lactation and Prolactin; (f) during lactation and P.M.S.

A.P.L., while the gonadotrophins of pituitary origin are without effect. It is interesting to note that all the hormonal preparations, especially P.M.S. and A.P.L., produced an intense luteinization of the ovaries; however, only P.M.S. was able to cause the formation of pregnancy corpora lutea. It can therefore be assumed that these large corpora lutea release hormonal substances either different from those secreted by the corpora lutea of lactation or in a different ratio. We tried to see whether the luteotrophic hormone, prolactin, would exaggerate the stimulation of the normally existing corpora lutea. We injected on the day following parturition for a period of 10 days a daily dose of 100 I.U. of prolactin. The growth of the young was normal if not better; the ovaries were slightly heavier (67 mg) than normal and the corpora lutea were well developed but not hypertrophied, as can be seen from Fig. 1. This is in accordance with another observation.[14]

If the small morphologic differences between the A.P.L.- and P.M.S.-treated rats are really associated with different hormonal secretions, the examination of the receptor organs should provide more information regarding the nature and even the level of the hormones in circulation. The vaginae in the 4 groups were mucified, indicating the presence of progesterone. The uteri presented, however, some interesting differences. In the case of the A.P.L.-treated rats the epithelium of the cuboidal type had an hillocky appearance. The stroma was dense, poorly developed and slightly edematous; the cells were darkly stained and fusiform. Glands were present. The general aspect was not much different

from that seen in untreated controls although in the latter case this organ was not enlarged. In the P.M.S.-treated rats, the uterine epithelium was tall, not degenerating and presented also many folds. The stroma was well developed and edematous with glands; the cells were large and vesicular with a tendency to being transformed into fusiform cells.

When these observations are compared with those of Atkinson and Hooker,[15] who used the modifications of the endometrium to estimate the level of estrogens and progesterone, it is suggested that in the A.P.L.-treated rats there is a low level of estrogens and progesterone, while on the other hand in the P.M.S.-treated rats there is a high level of estrogens and progesterone. If that be the case, it should be possible to inhibit lactation in ovariectomized lactating rats by giving estrogens and progesterone in a certain ratio; experiments are already in progress to prove that point.

Summary. The influence on lactation of various gonadotrophins of pituitary and chorionic origin has been studied in lactating rats. P.M.S. had a strong inhibitory effect resulting in the death of most of the young; A.P.L. had a slight effect on the growth curve of the young, while an anterior pituitary preparation was inactive. From histologic studies of ovary, vagina and uterus it is suggested that the activity of P.M.S. is due to a high level of estrogens and progesterone.

14 Fluhmann, C. F., and Laqueur, G. L., PROC. SOC. EXP. BIOL. AND MED., 1943, 54, 223.

15 Atkinson, W. B., and Hooker, Ch. W., *Anat. Rec.*, 1945, 93, 75.

16138

Antagonism of Sulfonamide Inhibition by Para-aminobenzoic Acid and Folic Acid in *Toxoplasma* Infected Mice.

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In feeding experiments[1] it has been demonstrated that sodium sulfathiazole and sodium sulfapyridine markedly reduce the fatality rate of mice infected with the protozoan parasite, *Toxoplasma*. Similar inhibitory action has been demonstrated for certain sulfonamide compounds in experimental monkey and avian malaria[2,3] and in experimental avian coccidiosis.[4,5,6]

The mode of action of sulfonamide compounds in the inhibition of multiplication of cells has been studied extensively. According to the Woods-Fildes theory sulfonamide compounds compete with the metabolite para-aminobenzoic acid and thereby interfere with cellular metabolism leading to the inhibition of multiplication of cells. Thus a high concentration of sulfonamide compound may lead to the prevention of cellular multiplication while on the other hand a high concentration of the metabolite can prevent such inhibitory action. This has been demonstrated to be true for various microorganisms both in cultures and in certain *in vivo* experiments. For example, the therapeutic action of sulfanilamide was completely nullified when administered with para-aminobenzoic acid to mice inoculated with *Streptococcus hemolyticus*[7] or

Diplococcus pneumoniae Type I.[8] In experimental avian malaria,[9,10,11] para-aminobenzoic acid has likewise been shown to have an antagonistic effect toward the therapeutic action of sulfonamide compounds.

Folic acid[12] and certain of its derivatives also have been shown to antagonize the bacteriostatic properties of sulfadiazine in *in vitro* experiments in which *Streptococcus faecalis* Ralston was used as a test organism. In view of this and the fact that folic acid contains a para-aminobenzoic acid moiety it might be assumed that similar results would be obtained in *in vivo* experiments with this compound.

The studies reported here were carried out to determine the effects of para-aminobenzoic acid, folic acid, sodium sulfathiazole and combinations of para-aminobenzoic acid and sodium sulfathiazole and folic acid and sodium sulfathiazole on experimental *Toxoplasma* infection in mice.

Methods. The parasite and maintenance of a stock strain. The R.H. strain of *Toxoplasma* used in all experiments was obtained through the courtesy of Dr. Joel Warren, Army Medical Center, Washington, D. C. in July, 1946. This organism has been maintained in mice by intraperitoneal inoculation of 0.2 ml of a 1×10^{-1} dilution of toxoplasma-containing peri-

1 Sabin, A. B., and Warren, J., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 19.

2 Coggeshall, L. T., *Am. J. Trop. Med.*, 1938, **18**, 715.

3 Levine, P. P., *Cornell Vet.*, 1939, **29**, 309.

4 Wehr, E. W., and Farr, M. M., *J. Parasitol.*, 1945, **31**, 359.

5 Farr, M. M., and Wehr, E. W., *J. Parasitol.*, 1945, **31**, 353.

6 Horton-Smith, C., and Boyland, E., *Brit. J. Pharm.*, 1946, **1**, 139.

7 Selbie, F. R., *Brit. J. Exp. Path.*, 1940, **21**, 90.

8 McCarty, M. M., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 133.

9 Maier, J., and Riley, E., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 152.

10 Marshall, E. K., Jr., Litchfield, J. T., Jr., and White, H. J., *J. Pharm.*, 1942, **75**, 89.

11 Seeler, A. O., Groessale, O., and Duesenberry, E. D., *J. Bact.*, 1943, **45**, 205.

12 Lampen, J. O., and Jones, M. J., *J. Biol. Chem.*, 1946, **164**, 485.

TABLE I.
Effect of Different Concentrations of Sodium Sulfathiazole and Para-aminobenzoic Acid on *Toxoplasma* Infection in Mice. Results in the table are based on *the last day in the experiment* on which an accurate LD_{50} titer could be calculated for the control animals.

Compound and conc. in diet	Proportion of inoculated mice dying at dilution of				LD_{50} titer on last day of exp.	Last day of exp.
	1x10⁻¹	1x10⁻²	1x10⁻³	1x10⁻⁴		
Sodium sulfathiazole 1.0%	0/7	1/7	1/6	0/4	—0.5	10
Control	7/7	6/7	7/7	3/7	3.93	10
Sodium sulfathiazole 0.15%	0/7	0/7	0/7	0/7	—0.5	9
Control	7/7	7/7	6/7	3/7	3.75	9
Sodium sulfathiazole 0.03%	6/6	2/6	0/6	0/6	1.74	7
Control	6/6	6/6	6/6	0/6	3.50	7
Para-aminobenzoic acid 3.5%	7/7	6/6	5/6	3/7	3.74	10
Control	7/7	6/7	7/7	3/7	3.93	10
Para-aminobenzoic acid 0.15%	5/6	6/6	6/6	3/6	3.83	8
Control	6/6	5/6	5/6	2/6	3.51	8

toneal fluid obtained from previously infected mice. Transfers of the strain have been made every 5 to 6 days.

Titration of the organism and inoculation of experimental animals. Toxoplasma-containing peritoneal fluid obtained from 4 stock mice on the fifth or sixth day of infection was pooled. Serial dilutions of 1x10⁻¹, 1x10⁻², 1x10⁻³ and 1x10⁻⁴ were prepared in Locke's solution. Six to 7 mice, weighing 15 to 20 g each, were inoculated with 0.1 ml of the first dilution and this procedure was repeated for each of the succeeding dilutions using a similar number of mice in each case. The animals were observed for a period of 15 days. Deaths which occurred within 3 days following inoculation are not included in the results. Mice which died between the fourth and fifteenth days were examined for the parasite and included in the data only if the parasite was observed in large numbers in the peritoneal fluid. The LD_{50} titer calculations were made according to the methods of Reed and Muench.[13]

Maintenance of mice during the test period. During the experimental period the mice were fed a standard diet which consisted of sucrose 3650 g, casein (vitamin free) 900 g, Crisco 250 g, salt mixture 150 g,[14] cellu flour Type

B 50 g, cod liver oil 75 drops, potassium iodide 75.6 mg, thiamin hydrochloride 25 mg, riboflavin 35 mg, calcium pantothenate 75 mg, niacin 70 mg, pyridoxine hydrochloride 25 mg, and choline chloride 5 g. Test compounds were administered to the animals in this diet. Approximately 4 g of diet or diet-drug mixture was provided for each mouse per day. For each drug tested one group of uninoculated mice was fed the diet-drug mixture thereby serving as a drug control. Water was provided at all times.

Results. Sodium sulfathiazole in a concentration of 1.0% in the diet protected 22 out of 24 mice inoculated with *Toxoplasma* for 15 days. One death occurred in the 1x10⁻² dilution group and one in the 1x10⁻³ dilution group. None of the inoculated mice treated with 0.15% sodium sulfathiazole succumbed to the experimental disease. At a concentration of 0.03% protection was slight although deaths occurred somewhat later than in the untreated controls. (Table I)

Toxic effects of sodium sulfathiazole were observed in mice fed a concentration of 1.0% of the drug. These were manifested as lethargy, ruffling of the coat and loss of weight. No toxic effects were noted in animals fed 0.15% or 0.03% sodium sulfathiazole.

Para-aminobenzoic acid at concentrations of 3.5% and 0.15% in the diet had no effect upon the experimental disease in mice. There

13 Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, 27, 493.
14 Wesson, L. G., *Sci.*, 1932, 75, 339.

TABLE II.

The Effects of Sodium Sulfathiazole and Para-aminobenzoic Acid, Separately and Combined, on *Toxoplasma* Infection in Mice. Results in the table are based on *the last day in the experiment on which an accurate LD$_{50}$ titer could be calculated for control animals.*

Compound and conc. in diet	Proportion of inoculated mice dying at dilution of				LD$_{50}$ titer on last day of exp.	Last day of exp.
	1x10^{-1}	1x10^{-2}	1x10^{-3}	1x10^{-4}		
Sodium sulfathiazole 0.15%	0/6	0/6	0/6	0/6	—0.5	8
Para-aminobenzoic acid 0.15%	5/6	6/6	6/6	3/6	3.83	8
Para-aminobenzoic acid + sodium sulfathiazole 0.15% each	6/6	6/6	5/6	0/6	3.39	8
Control	6/6	5/6	5/6	2/6	3.51	8

TABLE III.

The Effect of Sodium Sulfathiazole and Folic Acid Alone and in Combination on *Toxoplasma* Infection in Mice. Results in the table are based on the last day in the experiment on which the accurate LD$_{50}$ titer could be calculated for the control animals.

Drug and conc. in diet	Proportion of inoculated mice dying at dilution of				LD$_{50}$ titer on last day of exp.	Last day of exp.
	1x10^{-1}	1x10^{-2}	1x10^{-3}	1x10^{-4}		
Exp. I						
Folic acid 0.08%	6/6	6/6	6/6	3/6	4.00	9
Sodium sulfathiazole 0.08%	0/6	0/6	0/6	0/6	—0.5	9
Folic acid + sodium sulfathiazole 0.08% each	6/6	6/6	4/6	0/6	3.24	9
Control	6/6	6/6	6/6	2/6	3.74	9
Exp. II						
Folic acid 0.08%	6/6	5/6	5/5	1/6	3.29	8
Sodium sulfathiazole 0.08%	2/6	2/6	0/6	0/5	1.20	8
Folic acid + sodium sulfathiazole 0.08 % each	4/5	6/6	4/6	2/6	2.29	8
Control	6/6	6/6	6/6	3/6	4.00	8

was no evidence of toxicity resulting from the administration of this compound. (Table I)

When fed a diet containing both sodium sulfathiazole and para-aminobenzoic acid at 0.15% concentration each, the experimental disease in mice was not altered, *i.e.* these animals died at the same rate as untreated inoculated control animals. In this reversal experiment sodium sulfathiazole alone at 0.15% concentration afforded complete protection of all mice at all dilutions of the inoculum while 0.15% para-aminobenzoic acid failed to protect any of the mice. (Table II)

Two experiments were conducted to determine the effects of folic acid and sodium sulfathiazole singly and in combination on *Toxoplasma* infection in mice. A diet containing 0.08% of sodium sulfathiazole prevented the death of inoculated mice while a diet containing 0.08% of folic acid failed to protect any of the mice. When fed a diet

containing both drugs at 0.08% concentration each, mice were likewise not protected, *i.c.* animals so treated died at approximately the same rate as inoculated control animals. This experiment was repeated and similar results were obtained. (Table III)

Summary. Mice inoculated with fatal dosages of *Toxoplasma* (R.H. strain) were protected by the administration of sodium sulfathiazole in the diet in concentrations of 1.0%, 0.15%, and 0.08% but not by a concentration of 0.03%.

Neither para-aminobenzoic acid nor folic acid were observed to exert a significant effect on experimental toxoplasmosis in mice.

The protection afforded toxoplasma-infected mice by treatment with sodium sulfathiazole was nullified by the simultaneous administration of para-aminobenzoic acid or folic acid.

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Pharmacological Characteristics of Neohetramine, a New Antihistaminic Drug.* I.

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The symptomatic relief afforded by antihistaminic agents in the treatment of various allergic phenomena has stimulated the search for superior drugs. This search has led to the synthesis of Neohetramine,† 2-(N-dimethylaminoethyl-N-p-methoxybenzyl) amino pyrimidine monohydrochloride. Comparison with other antihistaminic drugs reveals that Neohetramine is relatively non-toxic, and is efficient both in counteracting the effects of histamine and in preventing anaphylactic shock. Preliminary details of this study are set forth below.

Experimental. Acute Toxicity. Acute toxicities of Neohetramine and other antihistaminic drugs were determined in mice (17 to 22 g males, C.F. 1 strain) and guinea pigs (300 to 400 g males). In both species signs of acute toxicity consisted of convulsions, complete extension of fore and hind limbs followed by respiratory paralysis and cardiac arrest. Necropsies performed daily for one week on surviving mice disclosed no gross abnormalities. The LD_{50} doses and associated limits of error, computed graphically,[1] are summarized in Table I. Neohetramine is the least toxic member of the series. In mice, the oral LD_{50} of Neohetramine is approximately twice as high as the intraperitoneal LD_{50}, and in guinea pigs, it is about 5 times as high.

Chronic Toxicity. Neohetramine was administered to 105 weanling rats‡ (approximately 20 per group) either by way of the diet (50, 100, 200 mg/kg daily) or subcutaneously (10, 20 mg/kg twice daily) for a

period of 91 days. The experimental animals and 21 (untreated) controls were weighed frequently and complete blood counts were taken at regular intervals. At the end of the test period the rats receiving Neohetramine were indistinguishable from their controls. Treated animals grew at a normal rate, exhibited no abnormalities in blood morphology,

TABLE I.
Acute Toxicity of Neohetramine.

Drug	LD_{50} mg/kg (base)	Limits of error (%)
Benadryl⁺	74.6	91-110
Pyribenzamine*	65.3	95-106
Hetramine*	61.0	91-110
Neohetramine*	119.0	96-105
'' †	245.0	91-110
'' † (guinea pig)	493.0	73-13?

* Dose given intraperitoneally.
† Dose given orally.

TABLE II.
Protection Against Intravenous Histamine.

Drug	TD_{50}* mg/kg (base)	Limits of error (%)
Hetramine	2.72	85-118
Neohetramine	1.15	64-147
Benadryl	1.00	74-135
Pyribenzamine	0.055	64-148

* Dose protecting 50% of the animals.

TABLE III.
Protection Against Nebulized Histamine.

Drug	TD_{50} mg/kg i.p.	Limits of error (%)
Hetramine	6.4	74-136
Benadryl	3.5	70-143
Neohetramine	3.5	73-140
Pyribenzamine	0.2	65-155
Neohetramine*	3.6	76-133
Pyribenzamine*	0.8	78-128

*Histamine and drug nebulized from same solution.

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* We are indebted to Mr. Bernard S. Rubin for his assistance.

† Neohetramine was formerly known as NH-188.

1 de Beer, *J. Pharmacol.*, 1945, **85**, 1.

‡ Rats were maintained on a stock diet of Purina Dog Chow and guinea pigs were maintained on Rockland Rabbit diet.

Antihistaminic drug*	Dosage (mg/kg i.p.)	No. survivals/ No. animals injected	% survival
None (controls)	—	4/31	12.9
Neohetramine†	5	8/19	42.1
	10	12/20	60.0
	25	10/20	50
Pyribenzamine†	10	13/20	65
	25	14/20	70
Benadryl	25	3/10	30

* Administered intraperitoneally 30 minutes before the shock injection of horse serum.
† The differences between Neohetramine and Pyribenzamine at the 10 and 25 mg dose levels, tested by the Chi Square Method, are not significant.

and developed no organ pathology. We are indebted to Professor George K. Higgins of the New York Medical College for the examination of all microscopic sections.

Protection against intravenously administered histamine. Groups of 10 male guinea pigs (300 to 400 g) were given graded intraperitoneal doses of the various antihistaminics. Thirty minutes later, a uniformly fatal dose (LD_{100}) of histamine diphosphate (0.5 mg per kg histamine) was injected intravenously with the results shown in Table II. In this test Hetramine was the least active, Neohetramine and Benadryl were about equally active and Pyribenzamine was the most active antagonist.

Protection against nebulized histamine. Groups of 10 guinea pigs (300 to 400 g males) were exposed, 4 at a time, to an atmosphere containing histamine aerosol.[2] In these experiments, 0.35 cc of a solution containing 12.5 mg histamine per cc was nebulized in 10 minutes into an 18 liter chamber. Untreated animals invariably died of typical histamine poisoning, but animals exposed 30 minutes after the intraperitoneal injection of increasing doses of the antihistaminic agents were protected as shown in Table III. When tested in this manner Neohetramine has the same potency as Benadryl. Pyribenzamine appears to be 17 times more active than Neohetramine but when, by a slight modification of procedure, histamine and antihistaminic drug

were nebulized from the same solution, Pyribenzamine was only 4-5 times as potent as Neohetramine.

Influence on Capillary Permeability. The ability of the various drugs to prevent histamine wheals was determined according to a modification of the method of Last and Loew.[3] Two concentrations of antihistaminic drug (1 and 4 micromols per cc) were combined with equal volumes of histamine in each of 7 concentrations (1, 2, 4, 8, 16, 32, and 64 micromols per cc). Randomized intracutaneous injections of 0.2 cc of each solution were made into the shaved abdominal skin of the rabbit and immediately afterward, 10 cc of 1% aqueous trypan blue was injected intravenously. Histamine antagonism was indicated by failure of the injected site to become blue. The end-point was taken as the highest concentration of histamine antagonized by each dose of antihistaminic drug. An analysis of variance performed on the data of quadruplicate assays indicated that differences attributable to animal variation were nonsignificant. Mean values are, therefore, presented. The data show that the effect of a solution containing 1.9 micromols of histamine per cc (*i.e.*, 0.21 mg) was completely nullified by the incorporation of one micromol of Neohetramine per cc (0.26 mg). In other words, one molecule of Neohetramine antagonized approximately 2 molecules of histamine. Under similar conditions one mole-

2 Loew, E. R., Kaiser, M. E., and Moore, J. *Pharmacol.*, 1945, 83, 120.

3 Last, M. R., and Loew, E. R., J. *Pharmacol.*, 1946, 89, 81.

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cule of Benadryl inhibited 1.5 molecules of histamine and one of Pyribenzamine nullified the action of nearly 9 molecules of histamine.

Excised tracheal tissue. Excised guinea pig tracheal tissue was studied according to the method of Castillo and de Beer.[4] In a 25 cc bath using Hastings-van Dyke solution with glucose, uniform contractions of the tracheal 'chains" produced by 0.72 μg of histamine per cc of bath fluid were reduced by incorporating in the histamine solution graded concentrations of Neohetramine (.029 to 1.876 μg per cc of bath fluid), or Pyribenzamine (.026 to 1.664 μg per cc). As shown in Figure 1, a good linear response was obtained with increasing concentrations of Neohetramine, whereas with Pyribenzamine the response was more erratic. Since the highest drug concentrations were equimolar and reduced the histamine contraction to the same extent (55%), Neohetramine and Pyribenzamine exhibited equal activity against this test object.

Anaphylaxis. The antianaphylactic activity of the various drugs was studied in 140 guinea pigs (300 to 400 g males). Approximately 2 weeks after intraperitoneal sensitization with one cc of 10% horse serum,[5] 27 of 31 untreated animals succumbed to an intravenous shocking dose of 0.5 cc of undiluted horse serum. Intraperitoneal injection of the antihistaminics 30 minutes before the shock dose resulted in survival as shown in Table IV. Protection was obtained with as little as 5 mg of Neohetramine per kg. When 10 mg per kg was injected 60% of the animals survived, but larger doses afforded no greater protection. Pyribenzamine exhibited the same order of activity as Neohetramine, and afforded no greater protection at higher dose levels. Benadryl afforded some protection (30%) at a dosage of 25 mg per kg but 50 mg per kg proved toxic to 3 out of 4 animals. Further experiments involving passive sensitization will be reported in the near future.

Discussion. Wherever possible, the foregoing data were recalculated in terms of the number of molecules of histamine antagonized by one molecule of Neohetramine; the ratios thus obtained varied from 0.5 to 9.0, indicating that the amount of histamine antagonized depended upon the test method employed. Depending on the type of tissue, the species

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In a clinical comparison of 2 antihistaminic drugs, Loveless[6] recently reported that Benadryl was better suited to the treatment of Ménières syndrome and intrinsic allergic asthma while Pyribenzamine was more effective in the treatment of non-seasonal extrinsic asthma. It may be inferred from this report that different drugs will be required in the treatment of different forms of hypersensitivity. Since laboratory methods of testing do not give a reliable index of the clinical value of the antihistaminic drugs, it would appear

that final evaluation of these drugs can be established only in human subjects, and then only with reference to the treatment of specific allergic states.

Summary. As judged by the intraperitoneal toxicity in mice, Neohetramine is about one-half as toxic as other antihistaminic agents. Weanling rats, receiving as much as 200 mg of Neohetramine per kg body weight per day over a period of 3 months, grew at a normal rate, exhibited no abnormalities in blood morphology and developed no organ pathology. Neohetramine showed marked activity against the bronchiolar and capillary actions of histamine. Quantitative estimates of the amount of histamine antagonized by Neohetramine varied with the method of testing. Neohetramine conferred protection against anaphylactic shock in guinea pigs actively sensitized to horse serum. The implications of these findings are discussed.

6 Loveless, M. H., *Am. J. Med.*, 1947, **3**, 296.

16140 P

Pharmacological Characteristics of Neohetramine, a New Antihistaminic Drug. II.

N. B. DREYER AND DONALD HARWOOD. (Introduced by John V. Scudi.)
From the Department of Pharmacology, University of Vermont, Burlington, Vt.

Several compounds which have anti-histaminic properties have been described recently.[1,2] Stress has been laid mainly on their ability to counteract histamine activity and certain allergic phenomena. Many of these compounds possess other properties as shown by their action on smooth muscle and glandular activity.[3,4] Neoantergan[3] is stated to have local anesthetic and quinidine-like properties. Benadryl[4] has some atropine-like activity.

Pyribenzamine[5] and 01013[6] enhance epinephrine responses. Described below are some effects of a new anti-histamine compound, Neohetramine.

Its anti-histamine activity was tested on the isolated ileum of guinea pig; isolated uteri of guinea pig, cat, and rat; blood pressure of cats and dogs; the intestine of the cat *in situ*, and blood vessels of the rabbit ear perfused at room temperature. Flow of saliva from submaxillary gland and blood pressure of the cat and dog served to test the effects of Neohetramine on the autonomic nervous system. All excised organs were suspended in

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3 Dews, P. B., and Graham, J. D. P., *British J. Pharmacol.*, 1946, **1**, 278.

4 Dreyer, N. B., and Denton, C., *Fed. Proc. Am. Soc. Exp. Biology*, 1947, **6**, 324.

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16141 P

Relation between Time of Fertilization and Follicle Cell Dispersal in Rat Ova.*

SAMUEL L. LEONARD, PRESTON L. PERLMAN† AND RAPHAEL KURZROK.

From the Department of Zoology, Cornell University, Ithaca, N.Y., and New York, N.Y.

Following the discovery that the enzyme hyaluronidase from sperm disperses the follicle cells of recently ovulated mammalian ova, a concept has arisen that the action of this enzyme facilitates fertilization by denuding the ova prior to or simultaneously with sperm entry.[1-4] The complete cell dispersal seen when mammalian ova are denuded by hyaluronidase *in vitro*[1,3] and the observation of Gilchrist and Pincus[5] who state "the freeing of adherent follicle cells is an inevitable accompaniment of sperm penetration..." have undoubtedly promulgated this concept. Observations to be reported in this note indicate no mass removal of the follicle cells prior to fertilization and that sperm penetration precedes the gross denudation of the ovum in the rat.

Methods. Female rats were bred and 12-26 hours later the ova were removed from the oviducts. The ova were examined in a depression slide for the disposition of the follicle cells and then transferred to a microscope slide to determine if fertilization had occurred. Hyaluronidase (0.1% solution) was drawn under the coverglass to remove the adhering follicle cells when it was necessary to observe the ovum proper.

In another experiment, 0.2 cc of hyaluronidase from bull or rat testes, in concentrations of 30-60 turbidity reducing units per cc, was introduced into each horn of the uterus of a rat in heat and the horns ligated near the cervix to prevent leakage. The enzyme was dissolved in either Ringer's solution or in the uterine fluid which was removed before administering the enzyme.

Experimental. In the first experiment, the ova of 9 rats were observed to be covered with follicle cells and remained in a compact mass when removed from the oviduct. They were similar in appearance to ova recovered from non-bred rats (100 cases). The ova proper could not be dissected free of their follicle cells with dissecting needles. After adding hyaluronidase, which removed the adhering follicle cells from the 65 ova recovered, spermatozoa were identified within the perivitelline space and polar bodies were present in every instance. These fertilized ova were obtained 12-16 hours post-coitus.

From 10 other rats, 31 fertilized ova were recovered, and they were either partially or completely denuded of their follicle cells. A jelly-like matrix surrounded the ova in several instances, but the follicle cells were few and scattered. Dead and motile sperm also were observed in this jelly. The denuded fertilized ova were recovered in two instances 13 hours post-coitus; the remainder at 16-26 hours.

Following the introduction of hyaluronidase into both uterine horns of 12 rats in heat, masses of ova were recovered in every instance 18-24 hours later. In not one case were the ova denuded. Addition of the uterine fluid of these rats to their own ova, *in vitro*, induced complete denudation in 10-20 minutes in all trials.

The results indicate that sperm make their way between the follicle cells and fertilize the ovum before any visible dispersal of the follicle cells occurs. Somewhat later the follicle cells are dispersed, freeing the ova. Furthermore, our observations indicate that

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oxygenated Ringer fluid kept at 36-37°C. and movements were recorded by the usual lever arrangement writing on smoked kymograph paper. Blood pressure was measured with a mercury manometer.

On the guinea pig ileum and uterus, and uterus of the cat where histamine caused contraction, Neohetramine was capable of abolishing the action of histamine. The amount of histamine causing a submaximal contraction varied from one experiment to another but was kept constant for any particular experiment. Histamine, as base, 0.03 to 0.3 μg per ml gave good contractions of guinea pig ileum. The amount of Neohetramine required to abolish such a contraction was determined and the ratio of Neohetramine to histamine base was calculated. For the cat uterus the ratio was .7:1; for guinea pig uterus, 1.4:1 and for guinea pig ileum, 2.7:1. Neohetramine showed greatest activity on the cat uterus, somewhat less on the guinea pig uterus, and least on the guinea pig ileum.

Neohetramine was less effective in counteracting the action of histamine on the blood pressure of the cat and dog. The fall in blood pressure caused by 1 to 2 μg of histamine was easily offset by 2.5 mg per kg of Neohetramine. However, the vaso-depression caused by larger amounts of histamine, (5-7 μg per kg) was reduced but could not be completely nullified by 8 to 10 mg per kg of Neohetramine. Neohetramine, like Pyribenzamine, was not capable of altering the inhibitory action of histamine on the rat uterus. The inhibition was as marked after Neohetramine as before, even when the ratio of Neohetramine to histamine was as high as 50 to 1. The vaso-constriction of the perfused rabbit ear caused by histamine could be completely counteracted by Neohetramine in a ratio of 1:1.

In atropinized cats and dogs under urethane, or chloralose and urethane anesthesia, Neohetramine (1-5 mg per kg) caused the following changes: an immediate drop in blood pressure without change in heart rate, followed by recovery in a few minutes. This was attributed to cardio-depressant action resulting from a high concentration of Neohetramine in coronary blood. As the drug became generally distributed in the body, the final concentration was insufficient to continue this cardio-depressant action. Large amounts of Neohetramine (8-10 mg per kg) produced a temporary hypotension. Straub heart preparations showed that Neohetramine diminished systolic contraction. As the concentration of Neohetramine in the perfusion fluid was increased, the depression of systole became greater. This was due to direct action on the cardiac muscle and was abolished on washing. Cardiometric measurements on the dog heart showed a diminished systolic and an increased diastolic volume after injection of Neohetramine. On the perfused rabbit ear, Neohetramine caused only slight transient dilatation.

Neohetramine showed little or no effect on sympathetic responses, following injection of epinephrine or sympathetic nerve stimulation. Potentiation of injected epinephrine as described for other anti-histaminics by Yonkman[5] and Lee[6] could not be demonstrated. This lack of potentiation to epinephrine was confirmed on the cat blood pressure and the isolated rabbit uterus. On the parasympathetic nervous system, Neohetramine exerted some atropine-like action on the chorda tympani, but even large doses (8 mg per kg) failed to abolish chorda secretion. Atropine on the other hand (.1 mg per kg) effectively abolished all chorda tympani activity. Vagal inhibition of the heart produced by weak faradization in the anesthetized cat could be eliminated by Neohetramine, but an increase in the strength of faradization tended to restore some vagal inhibition. However, Neohetramine did not lessen the vagal effects on the intestine. In several instances Neohetramine seemed to potentiate vagal contractions of the intestine.

Total and free acidities of gastric juice obtained by rhythmic stimulation of the left vagus were unaltered by Neohetramine, 1-5 mg per kg.

Summary. The above observations indicate that Neohetramine possesses well-marked anti-histaminic properties. In addition it has slight atropine-like actions; it does not alter epinephrine or sympathetic nerve responses.

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the bulk of the sperm and hyaluronidase remain in the uterus following copulation and no mass movement of the enzyme from the uterus into the tubes seems to play a part in the process of denudation and fertilization. We are of the opinion, however, that hyaluronidase is produced by those sperm which gain access to the tubes, and with the aid of this enzyme, the sperm traverse the follicle cell mass to fertilize the ovum. Later the concentration of enzyme is sufficient to denude the ova as was observed 24 hours post-coitus.

Conclusion. Fertilization of the rat ovum occurs before mass displacement of the surrounding follicle cells; denudation of the ova occurs subsequently. Since hyaluronidase, introduced into the uterus, does not pass into the tubes to denude the ova, it seems that only the enzyme associated with the sperm which reach the oviduct disperses the follicle cells.

16142

Influence of *M. varians* on Oral Infectivity of Mouse-Hamster (M-H) Virus.*

Vern Bolin and John A. Anderson

From the George B. Lockhart Memorial Pediatrics Research Laboratory, Department of Pediatrics, University of Utah Medical School, Salt Lake City, Utah

During the course of experiments on the susceptibility of white Swiss mice (Webster strain) to orally-administered M-H (Mouse-Hamster) virus, an unexplained reduction in the usual mortality of the mice occurred. During the period of January 5 to June 15, 1946 eight experiments involving 214 mice were conducted. The mortality to an orally-administered, highly concentrated mouse-brain suspension of the M-H virus in these 8 experiments varied between 48 and 80%, or an average mortality of 60%. Quite unexpectedly on July 26, the administration of the same dose of the M-H virus that had been used in the previous experiments now produced a mortality of only 20%. No explanation for this reduced mortality was available at that time as the virus end-point by intracerebral or foot-pad inoculation had not altered as compared with the previous 8 experiments. This reduced mortality to the M-H virus occurred again on December 21, 1946 (Experiments 12, 13, Table I). Thus, in the 13 experiments conducted during this year, there were only 2 in which the mortality

to the usual dose of the virus was less than 44%-80%, and these mortalities were only one-half to one-fourth of the lowest mortality previously achieved in the other 11 experiments. A review of the protocols of the 13 experiments and the method of preparation of the virus used in the oral feeding experiments shed some light on the problem.

Up to May 15th, consecutive passages of the M-H virus by intracerebral and foot-pad inoculation into the mouse had been performed until a seventh-passage virus was obtained on March 20, 1946. Contemplating a large number of experiments, subsequent to April, 1946, 200 mice were obtained and were inoculated subcutaneously into the foot-pad with a fatal dose of the seventh passage M-H virus. The brains were harvested when paralysis or death occurred, and a 25% emulsion in Ringer's solution was prepared. This total volume of the brain emulsion was then divided into 10 equal portions and stored in sterile lusterloid tubes in the CO_2 icebox at -40° C until needed in the future. Thus, the first experiment conducted with one of the samples of this new batch of virus was done in May, 1946, approximately 4 days after

TABLE I.
Mortalities of 13 Groups of Swiss Mice Fed M-H Virus Brain Suspension. The concentration of the virus suspension fed was 10% except in Exp. 4 and 5 when 2.5% was used. The virus end-point, by subcutaneous injection, in Exp. 1 to 11 was 100% of the animals when 0.03 cc of 10^{-6} dilution was given. In Exp. 12 and 13 the end-point was 10^{-7}.

| Exp. No. | Date | Virus | | | Mice | | | Mortality, % |
		Amt fed	Passage	No. of feedings	Age, days	Sex	No.	
1	1– 5–46	.5–.9	3	1	28	Mixed	26	65
2	25	.5–.9	4	1	180+	Males	50	48
3	2–13	.5–.9	5	1	50	Mixed	40	47
4	18	.5–.9	6	3	28	,,	25	80
5	18	.5–.9	6	3	28	,,	25	48
6	3–24	.5–.9	7	2	52	,,	10	60
7	5–19	.5	8	1	28	,,	18	77
8	6–15	.5	8	2	40	,,	20	55
9	7–26	.5	8	3	48	,,	20	20
10	9–21	.5	8	1	48	Males	20	45
11	21	.5	8	1	48	Female	27	44
12	12–21	.5	8	2	48	Males	28	17.8
13	21	.5	8	2	48	Female	20	10

the preparation of this batch of virus. The end-point of this fresh virus by foot-pad inoculation was still 10^{-6} and the mortality to the same large oral dose was 77% (Experiment 7, Table I). One month later on June 15, 1946, a second vial of the virus suspension was removed from the CO_2 icebox for the conduction of Experiment 8 (Table I). At this time the usual high mortality of 55% was obtained with the same dose and concentration of the M-H virus used previously. However, on July 26, 1946, a third tube of the virus suspension prepared in April was removed from the icebox for the conduction of Experiment 9 (Table 1). In spite of the fact that in this experiment the mice received 3 consecutive feedings of the same dose of virus as in previous experiments the mortality was only 20%. On Sept. 21, 1946, another vial was removed from the icebox for Experiments 10 and 11, and a good mortality was obtained. Again on Dec. 21, 1946, when Experiments 12 and 13 were conducted, mortalities of only 17.8% and 10% were obtained.

Fortunately, there was remaining from Experiments 12 and 13 (Table I) a small amount of the original 25% mouse-brain virus suspension that had been used in these 2 experiments. It was noted that this suspension had a rather orange or amber color and that particulate matter had settled out.

For this reason the material was cultured on various media, and an organism with the following properties was found. This organism tolerated a temperature of 60° C for 30 minutes. It reduced nitrates to nitrites and fermented all of the usual 6-carbon sugars, dextrins and glycerol with the production of acid only. Starch was not hydrolyzed. The inoculation of pure cultures of the organism intraperitoneally and intracerebrally into white mice produced no signs of peritonitis or meningitis. The mice remained well for the 14-day observation period subsequent to inoculation. The organism grew well on nutrient agar, the colonies having a faint yellow color when grown at room temperature, but not when grown at 37° C. When grown in a mouse-brain suspension in Ringer's solution at 4° C or 11° C the colonies at the meniscus have a faint pinkish tinge. The brain suspension also developed a slight orange-pinkish color. This organism has been identified as *Micrococcus varians*.

A relationship of this organism to the reduced oral infectivity of the M-H virus was postulated, and the following experiments were done.

An amount of eighth-passage virus, (that used in Experiments 12 and 13 and containing the *Micrococcus varians* sufficient to feed 18 mice was left. This was prepared as a 10% suspension and fed in 0.5 cc amounts for 2

the bulk of the sperm and hyaluronidase remain in the uterus following copulation and no mass movement of the enzyme from the uterus into the tubes seems to play a part in the process of denudation and fertilization. We are of the opinion, however, that hyaluronidase is produced by those sperm which gain access to the tubes, and with the aid of this enzyme, the sperm traverse the follicle cell mass to fertilize the ovum. Later the concentration of enzyme is sufficient to denude the ova as was observed 24 hours post-coitus.

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TABLE III.

Influence of *Micrococcus varians* on the Oral Infectivity of the M-H Virus.

	No. of mice	Mortality
Normal brain suspension	25	0
" " " + *Micrococcus varians*	25	0
M-H brain suspension	26	65.0
" " " + *Micrococcus varians*	24	33.3

TABLE IV.

Comparison of the Inhibiting Effect of *M. varians* Organisms and *M. varians* Culture Filtrate on the Oral Infectivity of the M-H Virus.

	No. of mice	Mortality, %
M-H virus + *M. varians* (incubated 97 days)	21	23.8
M-H virus + washed *M. varians* (incubated 48 hr)	20	35.0
M-M virus + *M. varians* filtrate (incubated 48 hr)	20	50.0
M-H virus only	17	59.4

successive days. These experiments presented in Table III demonstrated that when the M-H virus was incubated with a growing culture of the *Micrococcus varians* its oral infectivity was reduced by one-half.

. A virus titration of the specimen without bacteria was carried out by foot-pad inoculation at the time the brain samples were prepared and when fed. Likewise, the normal mouse brain and the normal mouse brain containing the *Micrococcus varians* were inoculated into 5 mice at the time of feeding. All mice survived.

At the time of the feeding of the virus, samples of brain suspension were removed from the bottom of each of the 4 tubes and cultured on individual blood agar plates. *Micrococcus varians* was found only in the 2 samples that had been inoculated 29 days previously.

After incubating for 29 days the sample of brain suspension containing the virus only and the sample of virus brain suspension which had been inoculated with the *Micrococcus varians* were titrated by foot-pad method in replicates of 5 each of 4-week-old mice. The volume of the dilution inoculated into each mouse was 0.03 cc. All samples of the M-H virus brain suspension had the same end point: a subcutaneous dose of 0.03 cc diluted to 10^{-7} killed 100% of the animals.

The following experiment was done in an attempt to determine whether or not this reduction in mortality was due to a direct action of the micrococcus on the virus and

thus to a reduction in the number of virus particles available for absorption, or perhaps due to the action on the virus of some agent in the culture filtrate produced by the micrococcus. Four groups of 8-week-old male mice were fed for 2 consecutive days 0.5 cc of the following 10% mouse brain virus suspension: (1) M-H brain-virus suspension plus micrococcus organisms (incubated at 4° C for 97 days). (2) M-H brain-virus suspension plus washed *Micrococcus varians* organisms (incubated at 4°C for 48 hours. (3) M-H brain-virus suspension plus *Micrococcus varians* filtrate (incubated at 4°C for 48 hours). (4) M-H brain-virus suspension only. These results are tabulated in Table IV.

Both the M-H virus suspensions containing the *Micrococcus varians* organisms had a lower mortality than that obtained with the M-H virus brain suspension alone. The filtrate from the *Micrococcus varians* culture did not significantly reduce the infectivity of the M-H virus. It appears that the action of the *Micrococcus varians* organism on the M-H virus is a direct one, possibly related to its metabolic activity or to some absorption phenomenon.

Summary. Suspensions of mouse brain tissue infected with the M-H (Mouse-Hamster) virus when incubated with a pure culture of the *Micrococcus varians* organism were found to have a markedly reduced oral infectivity for the white Swiss mouse. In spite of this reduction in oral infectivity there is no change in the infectivity of the virus material con-

Mice			Virus				
Sex	Age (days)	No.	Passage No.	Amt fed	No. feedings	Subcutaneous end point	Mortality, %
Mixed	32	18	8	0.5	2	10-6	16.6
,,	32	18	9	0.5	2	10-4	33.0

consecutive days. For controls, a small amount of this virus was passed subcutaneously into young Swiss mice whose brains were harvested in order to obtain a ninth-passage virus, free of the organism. Thus, a comparison of the original micrococcus containing eighth-passage virus, and micrococcus free ninth-passage virus could be made. The eighth-passage virus material produced a heavy growth of the micrococcus by aerobic culture while the ninth-passage virus material was sterile. A titration, by foot-pad, of each of the viruses was done, and a suitable group of animals, (18 in number), was fed an 0.5 cc dose of this ninth-passage virus as a 10% suspension.

Table II presents the results obtained in this experiment. The 18 male mice, fed on 2 consecutive days, a dose of 0.5 cc of the 10% eighth-passage virus, had a mortality endpoint by foot-pad inoculation of 10^{-6} and a mortality of only 16.6% to oral administration of the virus. On the other hand, the mice fed the same dose of ninth-passage virus, which had the higher oral infectivity, had a lower end point (10^{-4}) by foot-pad inoculation.

Table III presents the results of a more critical experiment in which the mortalities of Swiss mice fed the following preparations were determined: (1) 10% normal mouse brain suspension. (2) 10% normal mouse brain plus 1 cc of *Micrococcus varians* culture. (3) 10% M-H virus mouse brain suspension. (4) 10% M-H virus mouse brain suspension plus 1 cc of *Micrococcus varians* culture.

The method of preparation of the mouse brain suspensions for this experiment was as follows: 10% normal mouse brain in Ringer's solution was prepared. One hundred cubic centimeters of this solution was divided into 2 aliquots, each aliquot containing 50 cc. To one aliquot, 1 cc of a 25 day culture of the micrococcus was added. (This preparation of the *Micrococcus varians* was prepared from 20% guinea pig brain suspension in which the micrococcus had been inoculated and incubated for 25 days at 11° C). The second aliquot of the normal mouse-brain suspension received no *Micrococcus varians* inoculum. These 2 brain suspensions served as the controls. The brain suspensions containing the M-H virus were prepared by making 100 cc of a 10% M-H infected mouse-brain suspension. This volume was divided into 2 aliquots of 50 cc each. The first aliquot was inoculated with 1 cc of the *Micrococcus varians* culture while the second aliquot of the M-H brain suspension was not inoculated. All 4 aliquots were then cultivated, both aerobically and anaerobically, on blood agar plates at the time of preparation and all were found to be sterile except the 2 which had been inoculated with the micrococcus. These 4 specimens of mouse brain; namely, (1) normal mouse brain, (2) normal mouse brain plus *Micrococcus varians*, (3) M-H brain, and (4) M-H mouse brain plus *Micrococcus varians* were incubated at 11° C for 29 days. Ten days after the inoculation of the brain suspension with the *Micrococcus varians*, all of the tubes were cultured on blood agar. Growth existed only in the *Micrococcus varians* inoculated tubes. The other 2 control tubes were sterile. Twenty-nine days after inoculation with the micrococcus, the 4 tubes of mouse brain were fed to 4 groups of normal male mice. Each mouse was given 0.5 cc of the 10% mouse-brain suspension for three

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taining the micrococcus when it is injected subcutaneously. Filtrates of the micrococcus culture do not appear to inhibit the oral infectivity of the virus. It is suggested that the M-H virus may have been absorbed on the *Micrococcus varians* organism, and is thus not as readily available for invasion through the intestinal tract or that the virus itself may have been modified so that its oral infectivity has been reduced while its infectivity by subcutaneous inoculation is unchanged.

16143 P

Allantoin Clearance as a Measure of Glomerular Filtration Rate in Man.[*]

MEYER FRIEDMAN, SANFORD O. BYERS, AND PAUL M. ABRAHM.

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In a recent study,[1] the renal clearance of allantoin in the rat and dog was found to be equal to that of creatinine. This equivalence held true despite variations in both urine flow and plasma concentration of allantoin. It seemed probable therefore that the clearance of allantoin could serve as a measure of glomerular filtration rate in these two species. Furthermore, it seemed possible that the clearance of this same substance might serve as a measure of glomerular filtration rate in man. Therefore it was thought advisable to determine the renal clearance of allantoin in normal human subjects.

Methods. Five normal men and one woman received 10 g of allantoin dissolved in 500 cc of fresh orange juice by mouth at 8:00 A. M. on the day of the experiment. Each subject was kept at bed rest until 9:30 A. M. at which time they were catheterized and given 1000 cc of H_2O by mouth. At 10:00 A. M., the bladder was emptied, washed out with normal saline solution, a blood sample taken and the first urine collection begun. At 10:30 A. M., the bladder was emptied again, washed out with saline solution, and a second blood sample was obtained. The second urine collection began immediately after the bladder had been emptied and washed. At 11:00

A. M., the second and final collection period was terminated, and a third blood sample was obtained.

Urine and plasma samples were analyzed for allantoin by the method of Christman, Foster and Esterer,[2] modified as described in a previous study.[3]

The allantoin clearance of each subject then was calculated as an average of the clearances obtained for the 2 collection periods and corrected to 1.73 square meters of surface area.

Results. As Table I indicates, the allantoin content of plasma, 120, 150 and 180 minutes after oral ingestion of 10 g of this substance remained approximately constant. The average stability of the plasma allantoin during the entire clearance study of course eliminated any need for supplementary oral or parenteral administration of allantoin.

The average plasma allantoin clearance of the 6 subjects was found to be 123 cc per minute (See Table I). Individual clearances varied from 107 to 137 cc per minute. It should be mentioned that no ill effects were observed in any patient during or after the clearance study.

Discussion. Similar to the results obtained in the rat and dog,[1] the present study indi-

TABLE I.
The Renal Clearance of Allantoin in Human Subjects.

Patient	Age, yr	Avg urine flow (cc/min)	Plasma allantoin conc. (mg/100 cc)			Renal allantoin clearance (cc/min)		
			10:00	10:30	11:00	Per. I	Per. II	Avg
W.J.	45	13.45	7.4	7.0	7.5	101	113	107
H.B.	22	3.70	6.1	7.1	6.8	124	136	130
G.C.*	43	10.00	5.5	5.1	4.3	134	140	137
H.S.	43	5.70	6.9	7.7	8.8	132	114	123
R.S.	42	5.20	5.3	4.4	5.1	115	114	127
D.C.	32	5.90	4.3	4.3	4.8	131	123	115
Avg		7.33	5.9	5.9	6.2	123	123	123

* Female.

cates that the clearance of allantoin probably measures the rate of glomerular filtration in man. Thus, the average allantoin clearance obtained in the 6 subjects (123 cc per minute) is approximately the same as the value found for the inulin clearance (123 cc per minute) in man by Smith, Goldring and Chasis[4] and by one of us (M.F.) in a previous study.[5]

Conclusion. The average renal clearance of allantoin in 6 normal human subjects was found to be 123 cc per minute. The similarity of this value to that now accepted for the renal clearance of inulin (a glomerular filtrate without tubular reabsorption) strongly suggests that the renal clearance of allantoin is at the level of glomerular filtration.

[4] Smith, H. W., Goldring, W., and Chasis, H., *J. Clin. Invest.*, 1938, **17**, 263.

[5] Friedman, M., Selzer, A., and Rosenblum, H., *J. A. M. A.*, 1941, **117**, 92.

<div align="center">16144</div>

Effect of Folic Acid* and Bis (β-Chloroethyl) Sulfide (Mustard Gas)† on Transplanted Mouse Lymphosarcoma.‡

Allan D. Bass and Marion L. H. Freeman.

From the Department of Pharmacology, Syracuse University College of Medicine, Syracuse, N. Y.

Leuchtenberger *et al.* [1,2] have shown that folic acid is a tumor growth inhibitor for sarcoma 180 and for spontaneous mammary

tumors in mice. Bass and Freeman[3] were not able to demonstrate a similar regression in transplanted lymphoma 6C3HED in mice. The present investigation was designed to further study the effect of folic acid on the 6C3HED tumors, when administered alone or in combination with bis (β-chloroethyl) sulfide. It was hoped that the folic acid might reduce the severity of the toxic side effects of bis (β-chloroethyl) sulfide. Although this did not prove to be the case, certain interesting observations were made which are reported in this paper.

* The "synthetic folic acid," "Folvite," and "Teropterin" used were supplied by Lederle Laboratories, Pearl River, N.Y.

† The bis (β-chloroethyl) sulfide was supplied by Edgewood Arsenal, Md.

‡ These studies have been made with the assistance of a grant-in-aid from the National Cancer Institute.

[1] Leuchtenberger, C., Lewisohn, R., Laszlo, D., and Leuchtenberger, R., Proc. Soc. Exp. Biol. and Med., 1944, **55**, 204.

[2] Leuchtenberger, R., Leuchtenberger, C., Laszlo, D., and Lewisohn, R., *Science*, 1945, **101**, 46.

[3] Bass, Allan D., and Freeman, Marion L. H., *J. Nat. Cancer Inst.*, 1946, **7**, 171.

Methods. Adult C_3H mice of both sexes obtained from Jackson Memorial Laboratories at Bar Harbor, Maine and Lymphosarcoma $6C_3HED$ were used. Small fragments of tumor were implanted subcutaneously into the right axillary region with a 16-gauge needle. When the tumors were large enough to measure, treatment was started. Ninety animals were used for this study; 30 receiving bis (β-chloroethyl) sulfide alone, 30 synthetic folic acid alone, and 30 both the bis (β-chloroethyl) sulfide and folic acid.§ Folic acid was administered in daily doses of 0.1 mg per mouse. The bis (β-chloroethyl) sulfide was given in doses of 3 mg per kg at 48-hour intervals unless the animals developed toxic symptoms in which case the interval was prolonged or the dose reduced to fit the individual case. Diarrhea and/or a weight loss of 1 g or more in 24 hours were used as criteria for varying the procedure until cessation of such symptoms. Both folic acid and bis (β-chloroethyl) sulfide were administered intraperitoneally; folic acid in a phosphate buffer solution of pH 7.1 and bis (β-chloroethyl) sulfide in 0.076% by volume solution in propylene glycol. All animals were fed Purina dog chow *ad libitum.*

Results. Fig. 1 summarizes the results obtained.
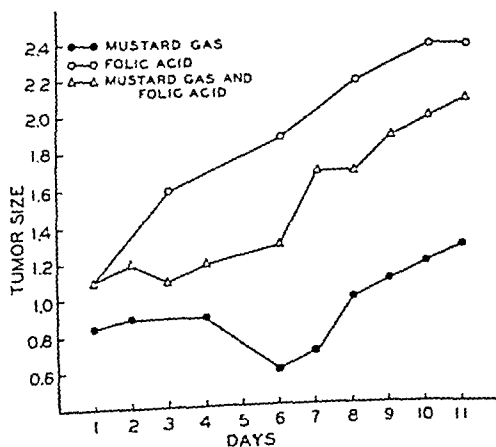
FIG. 2.

Graph to show the growth rate of $6C_3HED$ tumors in control animals as compared with animals receiving "synthetic folic acid." Propylene glycol and phosphate buffer were given intraperitoneally to 10 animals each. Thirty animals received folic acid.

Since bis (β-chloroethyl) sulfide is lethal for some animals at the dosage used, the points plotted on the curve represent the average tumor size of surviving animals. Tumor size is indicated as one half the length plus the width expressed in centimeters. Tumors of animals receiving bis (β-chloroethyl) sulfide showed a diminished rate of growth with a definite period of regression on the sixth day after the treatment was begun. Tumors of animals receiving both folic acid and bis (β-chloroethyl) sulfide clearly showed less effect of the therapy than when bis (β-chloroethyl) sulfide alone was administered. Although less regression or growth inhibition was evident in the animals receiving both bis (β-chloroethyl) sulfide and folic acid the survival time was not significantly altered. The rate of growth of the tumors in control animals receiving phosphate buffer or propylene glycol was approximately the same as the tumor growth rate of mice receiving folic acid (Fig. 2).

To determine whether there was any difference in growth rate of lymphoma $6C_3HED$ when tumor-bearing mice were given pteroyl mono-glutamic acid or pteroyl tri-glutamic acid, we gave 15 C_3H mice 0.1 mg of "Folvite" daily in phosphate buffer and another 15 mice 0.1 mg of Teropterin (pteroyl tri-

FIG. 1.

The indicated treatment was begun on the first day shown in graph. This is approximately 7 days after transplantation.

§ Unless otherwise stated, folic acid in this paper will refer to "synthetic folic acid" (Lederle).
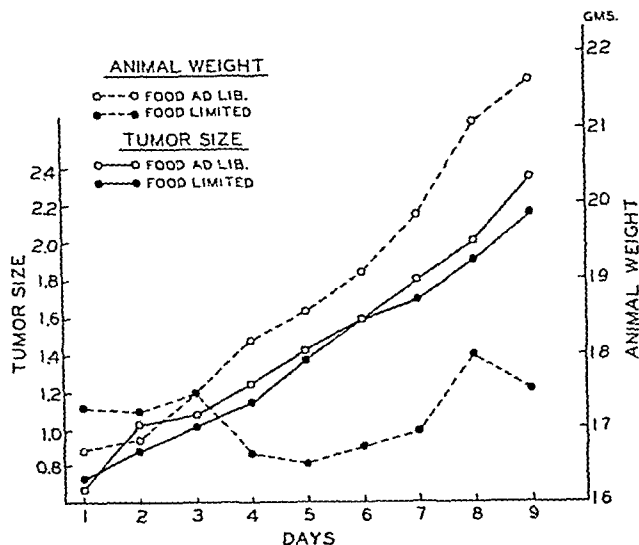
FIG. 3.
Food limitation was begun on the second day.

glutamic acid) daily for 5 days. The growth curve of the tumor in these two groups was not significantly different from controls treated with phosphate buffer alone, or from the growth curves when "synthetic folic acid" was used.

The weights of the animals receiving both bis (β-chloroethyl) sulfide and folic acid were maintained better than those receiving bis (β-chloroethyl) sulfide alone. To exclude the possibility of weight loss associated with drug administration as the cause for the differences in tumor regression a paired feeding experiment was carried out, the results of which are shown in Fig. 3. Here it is shown that limitation of food to 60% of that eaten

by the control mice has no measurable effect upon the tumor growth. The weight loss produced by the food limitation is comparable to that which is frequently encountered after bis (β-chloroethyl) sulfide administration. Additional studies are necessary to elucidate the mechanism of folic acid antagonism to bis (β-chloroethyl) sulfide.

Summary. "Synthetic folic acid", "Folvite," or "Teropterin" in the dosage employed do not produce regression in transplanted 6C₃HED tumors in C₃H mice. Administration of synthetic folic acid partially inhibits the effect of bis (β-chloroethyl) sulfide on lymphosarcoma 6C₃HED.

Activation of Staphylocoagulase.*

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Smith and Hale[1] have shown that the in-

ability of staphylocoagulase to clot citrated plasmas is, in most cases, due to absence of an "activator substance" for the staphylo-

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coagulase. Gratia[2,3] originally showed that staphylocoagulase clotting is independent of prothrombin activation, and Rigdon's[4] recent evidence, using heparin, confirms the inability of antithrombic agents to inhibit staphylocoagulase clotting. We have repeatedly confirmed these facts and present the following data as a preliminary report of progress in identifying the "activator" in various plasma, serum, and other materials.

Materials and methods. 1. STAPH.: Staphylocoagulase was prepared by precipitating 48-hr. *Staphylococcus aureus* (stock strains) broth-culture filtrate (Berkefeld N), or centrifugate, with 3 volumes of 95% ethyl alcohol, at 0°C. The method is essentially that by which Tillett and Garner[5] prepared "fibrinolysin" (*streptokinase[6]*). The ability to obtain an active staphylocoagulase from simple broth culture filtrates confirms Smith and Hale[1] and opposes Lominski.[7] whose results were negative unless plasma was added to the culture medium. A typical yield from 850 cc broth culture filtrate approximates 1.5 g of grayish-brown powder, completely soluble in 1% solution in our citrated borate buffer. The powder, stored in refrigerator (5°C) for 8 months, showed no loss of activity and its solutions were stable for as long as 10 days at room temperature. Reagents previously described:[6] 2. buff. (cit.): borate buffer (plus 0.4% sod. citrate; pH: 7.55), 3. B.F.: (Armour's) bovine plasma fraction-I (50% fibrinogen), 4. H.F.: (Harvard) human plasma fraction-I (35% fibrinogen).‡ 5. PRO.: (Seegers') purified bovine prothrombin.

Additional reagents: 6. H.Pl.: human plasma, 'Lyovac' dried (Sharp and Dohme), 7. B.f.: bovine fibrinogen (Seegers[8]), S. Ac.G.: "accelerator globulin" from bovine plasma, courtesy Dr. W. H. Seegers[9] (Wayne Univ.), 9. Factor V: (after Owren[10]) prepared from dog plasma, 10. H.Pl.G. and H.S.G.: human 'globulin,' fractionated by 50% sat. $(NH_4)_2SO_4$ from (a) H.Pl. (v. 6) and (b) serum from same after clotting with $CaCl_2$, 11. H.Pl.A. and H.S.A.: human plasma (a) and serum (b) 'albumin,' fractionated from supernatants of above at 100% sat. $(NH_4)_2SO_4$, 12. H.S.A. cryst.: crystalline human serum albumin (Harvard Labs.),‡ 13. tpln.: commercial thromboplastins— I. Difco, II. Maltine, prepared according to directions.

Results. Table I describes staphylocoagulase tests with human plasma and the globulin and albumin fractions from plasma and serum. Bovine fraction-I (fibrinogen), plasma, and buffer (without STAPH.) do not clot (2) and B.F. has no activator substance (3), which must be supplied by the addition of plasma (1). The globulins from plasma (5) or serum (9) contain a weak thrombin, but the definite clotting improvement in the presence of staphylocoagulase (4,8) indicates some activator substance also. Crude albumins from plasma (6) or serum (10) are the best single source of activator substance, and are not coagulant in the absence of staphylocoagulase (7,11). Further investigation of these albumin fractions is currently being undertaken to yield more information on the staphylocoagulase mechanism.

Table II lists other substances tested by the same means as in Table I, except for

1 Smith, W., and Hale, J. H., *Brit. J. Exp. Path.*, 1944, **25**, 101.

2 Gratia, A., *C. R. Soc. Biol.*, 1919, **82**, 1245, 1247, 1393.

3 Gratia, A., *ibid.*, 1920, **83**, 584, 585, 649, 1221.

4 Rigdon, R. H., and Haynes, A., *Ann. Surg.*, 1942, **116**, 430.

5 Tillett, W. S., and Garner, R. L., *J. Exp. Med.*, 1933, **58**, 485; 1934, **60**, 239.

6 Ferguson, J. H., Travis, B. L., and Gerheim, E. B., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 285, 302.

7 Lominski, I., *Nature*, 1944, **154**, 640.

‡ The human plasma fractions used in this work were prepared from blood collected by the American Red Cross under a contract between the Office of Scientific Research and Development and Harvard University, and supplied through the courtesy of Drs. Cohn, Edsall, Minot, and colleagues.

8 Ware, A. G., Guest, M. M., and Seegers, W. H., *Arch. Biochem.*, 1947, **13**, 231.

9 Ware, A. G., Guest, M. M., and Seegers, W. H., *J. Biol. Chem.*, 1947, **169**, 231.

10 Owren, P. A., *The Coagulation of Blood: Investigations on a New Clotting Factor* (Oslo), 1947, p. 327.

TABLE I.

Staphylocoagulase Tests with Plasma and Serum Fractions.

No.	B.F. (1%) (0.5 cc)	Buff. (cit.) (0.25 cc)	Staph.† (0.25 cc)	Plasma fraction (0.5 cc)	Clotting-times (min., at 39°C)* 1+	2+	3+	4+
1	+	—	+	H.Pl.	21'	78'	—	107'
2	+	+	—	H.Pl.	No clotting			
3	+	+	+	—	,,	,,		
4	+	—	+	H.Pl.G.	13'	—	78'	163'
5	+	+	—	H.Pl.G.	40' (clot never complete)			
6	+	—	+	H.Pl.A.	21'	—	33'	46'
7	+	+	—	H.Pl.A.	No clotting			
8	+	—	+	H.S.G.	—	—	21'	42'
9	+	+	—	H.S.G.	—	—	21'	92'
10	+	—	+	H.S.A.	—	—	—	17'
11	+	+	—	H.S.A.	No clotting			

† Staph.: Lot No. 5-6.
* Degree of clotting expressed as follows:
    1+: first appearance of weak clot;
    2+: weak clot with considerable liquid;
    3+: good clot with a little liquid but too soft to invert tube;
    4+: solid clot, tube invertible.

TABLE II.

Tests for "Activator Substance" of Staphylocoagulase.

No.	Material tested (0.5 cc)	B.F. (1%) (0.5 cc)	Staph.† (0.25 cc)	Clotting-times (39°C) 1+	4+
1	H.S.A., cryst. (1%)	+	+	No clotting (48 hr)	
2	PRO. (1%)	+	+	,, ,, ,,	
3	Factor V (1%)	+	+	,, ,, ,,	
4	Ac.G. (1%)	+	+	,, ,, ,,	
5	H.F. (1%)*	—	+	20'	40'
6	B.f. (1%)*	—	+	40'	overnight
7	tpln—I (5%)	+	+	—	240'
8	tpln.—II (2.5%)	+	+	—	-60'

† Staph.: Lot No. 5-4.
* Double volumes (1.0 cc) of H.F. or B.f. used, and B.F. omitted.

substituting the respective material for the plasma fraction. The negative results with (1) *crystalline* human serum albumin, (2) purified prothrombin, and the recently identified globulin accelerators of prothrombin conversion (thrombic clotting), *viz.* (3) Owren's factor V and (4) Seegers' Ac.G., are noteworthy. The impure fibrinogens (5,6), which contain some activator substance, should be compared, in this respect, with B.F. (Table I, 3). With enough citrate in our test system to prevent possible thrombin formation (only negligible traces of prothrombin occur in B.F.,[6] however), the evidence for accelerator substance in the thromboplastins (7,8) is interesting.

Summary. The preparation and some properties of a stable staphylocoagulase are described, together with data to indicate that the "activator substance" which it needs in order to coagulate fibrinogen can be provided by certain plasma protein fractions, particularly being associated with the albumins.

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S. F. QUAN, L. E. FOSTER, A. LARSON, AND K. F. MEYER.

From the George Williams Hooper Foundation, University of California, San Francisco, Calif.

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strain (Otten)[3] by 4 units/cc, while in the presence of 10% blood serum, 40 units/cc were required to kill 10,000 virulent *P. pestis* "Yreka" in 48 hours. Other factors, such as the chemical composition and the pH of the suspending medium, the concentration of the antibiotic, and the age and density of the culture, influence the bactericidal activity of streptomycin on *P. pestis*. 5,000 µg/cc in broth destroyed 22,000 million virulent *P. pestis* (Yreka)/cc, while the same concentration sterilized 33,000 million/cc in physiologic saline. Four virulent strains trained to resist streptomycin in the amount of 5,000 µg/cc were avirulent and resisted only 2,500 units/cc dihydrostreptomycin.

(2) *Effect on mice and guinea pigs infected subcutaneously.* The relative sensitivity of *P. pestis* to streptomycin permits effective therapy of experimental bubonic disease caused by 100 to 1,000 multiples of the M.L.D. in highly susceptible (ABC) mice. Treatment usually begins on the 48th hour after subcutaneous introduction of the bacilli, when infection is generalized, a bacteremia is well established in 40 to 60% of the animals, and the immunity mechanism is partially damaged by toxins. Irrespective of dosage or frequency of administration, sulfonamides save an average of only 35% of mice at this stage of infection. In 70 separate experiments, treating as many groups of 20-50 mice with varying amounts of streptomycin, advanced experimental bubonic plague was completely cured with 500 µg/3 hours for 3 days, or a total of 12,000 µg (12.0 mg). The median effective dose was 1,000 to 1,250 µg for a 20 g mouse, or 50 to 62 µg/g when one intraperitoneal injection was given on the 48th hour of infection. Bacteriological autopsies demonstrated that on the 14th hour after injection of the antibiotic,

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Bubonic plague infections which have progressed to an extent comparable to 48-hour infections in mice occur in guinea pigs 120 hours after subcutaneous injections of 1,000 multiples of the M.L.D. With a dose of streptomycin of 20,000 μg/kg or approximately 10 mg per day, a total of 100 mg over a period of 10 days, 80 to 100% of the 40 guinea pigs used in experiments were cured. It requires less streptomycin to cure guinea pigs in a stage of mild septicemia than to cure mice, on the basis of units of antibiotic to grams of body weight. The administration of 25,000 to 50,000 μg/kg in 3 doses, or a daily injection of 37,500 to 75,000 μg/500 g guinea pig of

the early streptomycin preparations proved toxic, but recent lots of the antibiotic in the same doses are well tolerated.

In the tests under consideration and those of Wayson and McMahon[4] persistence of plague bacilli in regional buboes despite large doses of the antibiotic suggested local chemotherapy. Guinea pigs with well-developed buboes on the 120th hour after subcutaneous administration of 100,000 *P. pestis*, were injected around the local swelling with 12,500 μg in 0.5 cc of physiologic saline at 12-hour intervals for 10 days. All were cured and plague lesions were sterilized. Sodium sulfamerazine administered in the same manner in the amount of 500 mg/kg/12 hours was inhibitory but not bactericidal, and cured no more than 40% of the infected animals. Local injections of streptomycin proved very irritating, and even though buboes became rapidly sterile, the necrotic areas were much larger than those of controls or of animals treated systemically.

(3) *Effect on septicemic plague in mice.* To establish the maximum effectiveness of streptomycin, a rapidly progressive *septicemic* infection was produced in slightly-resistant Swiss mice by intraperitoneally injecting 2,000 *P. pestis*. Bacteremia and toxemia exist in such mice by the 9th-12th hour after infection, and death takes place in 34 to 72 hours. In some respects, this infection model resembles the clinical form of human septicemic plague induced by direct blood stream infection through several fleabites. Repeated tests of the kind shown in Fig. 1 conclusively demonstrated that 2 to 3 large doses of 400 to 800 μg at 3-hour intervals, or a total of 1,200 to 1,600 μg, cured 80 to 90% of nonbubonic septicemic plague infections. Small doses merely delay death. If treatment is postponed until the 18th or 24th hour, 2 doses of 800 μg each cure only 25 to 45% of infected mice. Only early treatment with large doses of streptomycin cures septicemic plague in mice. Sulfadiazine and highly potent antiplague serum proved ineffective under

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(3) *Effect on septicemic plague in mice.* To establish the maximum effectiveness of streptomycin, a rapidly progressive *septicemic* infection was produced in slightly-resistant Swiss mice by intraperitoneally injecting 2,000 *P. pestis*. Bacteremia and toxemia exist in such mice by the 9th-12th hour after infection, and death takes place in 34 to 72 hours. In some respects, this infection model resembles the clinical form of human septicemic plague induced by direct blood stream infection through several fleabites. Repeated tests of the kind shown in Fig. 1 conclusively demonstrated that 2 to 3 large doses of 400 to 800 μg at 3-hour intervals, or a total of 1,200 to 1,600 μg, cured 80 to 90% of nonbubonic septicemic plague infections. Small doses merely delay death. If treatment is postponed until the 18th or 24th hour, 2 doses of 800 μg each cure only 25 to 45% of infected mice. Only early treatment with large doses of streptomycin cures septicemic plague in mice. Sulfadiazine and highly potent antiplague serum proved ineffective under

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[4] Wayson and McMahon, *J. Lab. and Clin. Med.*, 1946, **31**, 323.

DYNAMICS OF STREPTOMYCIN ON EXPERIMENTAL SEPTICEMIC PLAGUE IN MICE
(INTRAPERITONEAL INFECTION)
EXPERIMENT 97
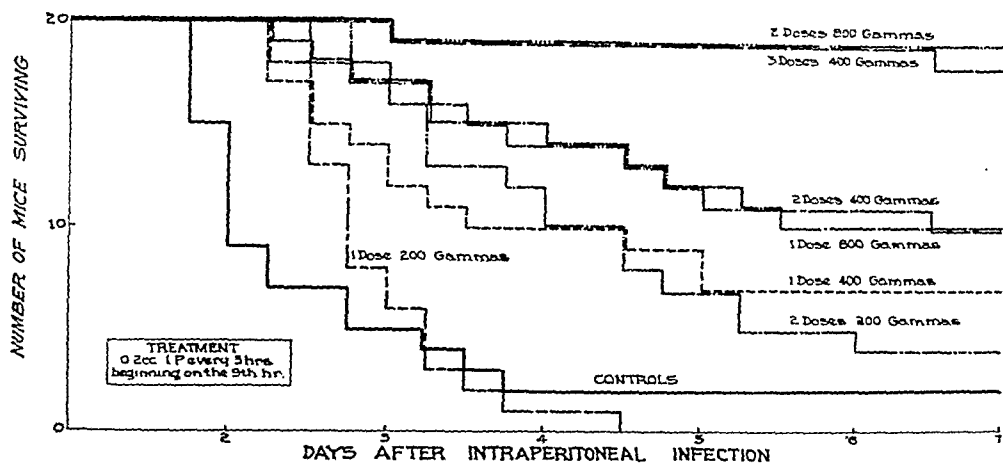
FIG. 1.

STREPTOMYCIN IN THE THERAPY OF THE 36·HOUR INTRANASAL PLAGUE
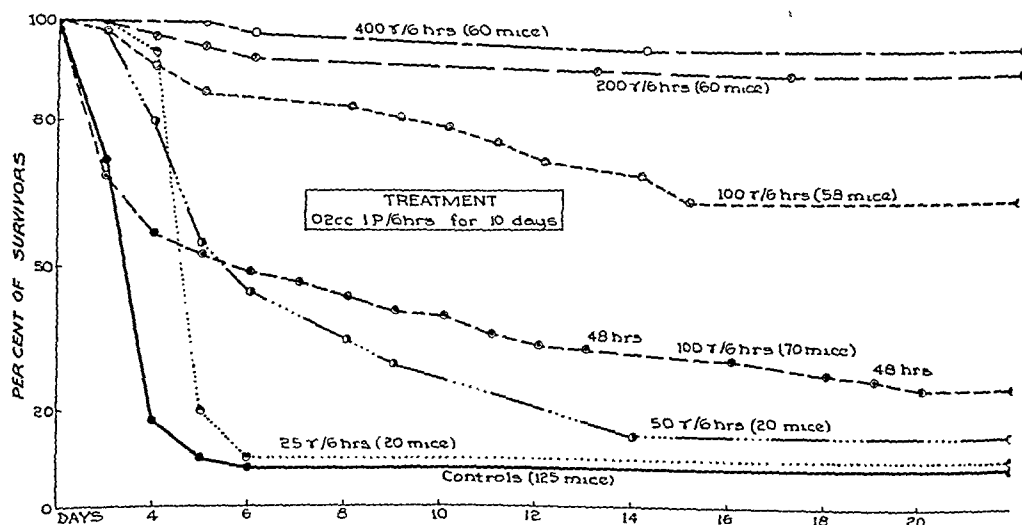
FIG. 2.

identical experimental conditions. There is at present no evidence that sulfonamides synergistically enhance the remedial action of streptomycin. On the other hand, numerous experiments furnished data that potent antiplague serum administered simultaneously with small doses of streptomycin increased the percentage of cures. Finally, active immunization with plague antigens potentiates the ac-

tion of streptomycin both in mice and in guinea pigs and the partial immunity so gained allows use of smaller doses of streptomycin.

(4) *Effect on pneumonic plague in mice.* Pneumonic plague infections may be produced in mice by intranasal instillation of 2,500–5,000 *P. pestis* in 0.05 cc of saline; the lobular lesions recognizable on the 36th hour after infection contain several million

INTRANASAL PLAGUE TREATED WITH SERUM AND SULFADIAZINE
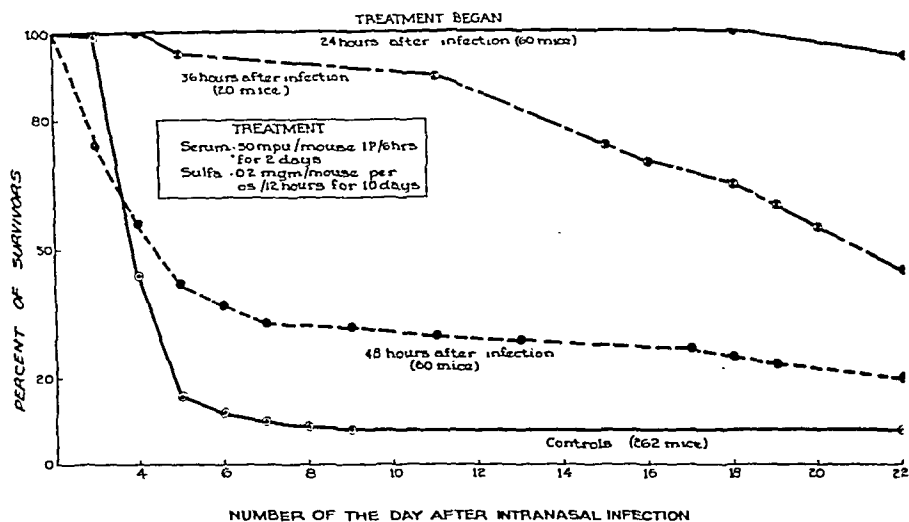
FIG. 3.

BACTERIOLOGICAL STUDY OF INTRANASALLY - INFECTED STREPTOMYCIN - TREATED MICE
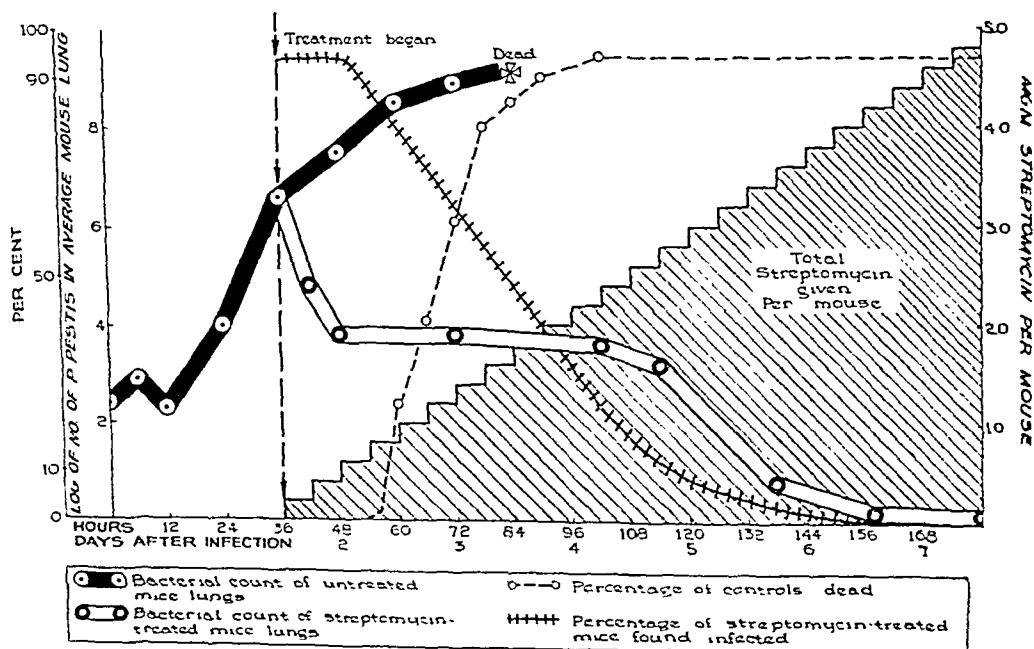
FIG. 4.

plague bacilli. As might be expected, 200 to 400 μg of streptomycin hydrochloride or sulfate, given every 6 hours, effectively cure 90 to 95% of the infections. Smaller doses or delayed treatment reduces the chance for cures (Fig. 2). Sulfadiazine in combination with antiplague serum is less effective than streptomycin (Fig. 3). The remarkable bactericidal action of streptomycin is fully documented by periodic bacteriological autopsies of treated and untreated mice (Fig. 4). Six hours after treatment with 200 μg had begun, the number of plague bacilli found in the lungs of the treated mice was reduced to approximately 60,000, while in untreated animals it had advanced to 10,000,000. By the 12th to 24th hours of treatment, the spleen became sterile and 5000 or less organisms were counted in the lungs. By the 96th hour after infection, when all untreated mice had died, the lungs and bronchial lymph nodes of treated mice were either sterile or contained only a few thousand plague bacilli in the abscess-like patches of pneumonia. No plague bacilli have been isolated from lungs or lymph nodes 100 hours after treatment with streptomycin. These results fully attest to the remarkable therapeutic efficacy of a total of 5 mg of streptomycin in experimental plague of mice. They justify an expectation that it will be equally effective in human pneumonic plague if administered early and in adequate dosage.

(5) *Suggested schedule of treatment in human plague.* Streptomycin is the most effective therapeutic agent thus far discovered for the treatment of bubonic, septicemic and pneumonic experimental plague infections in mice and guinea pigs. It is recommended that human plague be treated as soon as diagnosed with daily doses of 2 g of streptomycin in bubonic plague, and 4 to 6 g in the septicemic and pneumonic diseases; injections should be given at 4-6 hour intervals for the first 2 days. The dose may then be reduced, but in order to prevent clinical recurrences treatment should be continued for at least 8 days on a one g level or substituted with adequate sulfadiazine therapy. In profound toxemia, simultaneous administration of a potent antiplague serum, to assist the immunity mechanism, may prove beneficial.

Summary. Streptomycin in the amounts of 0.4 to 4.0 μg/cc is bactericidal for different strains of *P. pestis* in 5 days. Advanced experimental bubonic plague in mice is completely cured with 500 μg/3 hours for 3 days, or a total of 120 mg. Between 80 to 90% of the mice in a state of septicemic plague may be saved with a total of 1,200 to 1,600 μg. The remarkable bactericidal action of streptomycin is best demonstrated on experimental pneumonic plague; 5 mg of the antibiotic sterilize lungs and lymph nodes within 100 hours after treatment has been instituted. It is recommended that human plague be treated as soon as diagnosed with 2 to 4 g of streptomycin daily depending on the state of infection.

16147

Distribution of P³² in Incubated Egg.*

FRANK J. DIXON. (Introduced by Shields Warren.)
*From the Laboratory of Pathology of the Harvard Cancer Commission,
Harvard Medical School, Boston*

As part of our observations of incubated eggs injected with P³² we traced the course of the isotope within the components of the egg throughout the incubation period. The phosphate content of a hen's egg of this size is approximately 220 mg[1] and the amount

of phosphate injected in this procedure was 3.5 mg.

Materials. Eggs—White Leghorns average weight 60 g incubated at 38°C with 70-80% relative humidity. P³² as KH_2PO_4—a 10% solution of the salt in H_2O was used for injection. The P³² was supplied as KH_2PO_4 by Clinton Laboratories, U. S. Government, Oak Ridge. Tenn.

Procedure. Approximately 0.05 cc of 10% KH_2PO_4 solution containing 20 μc per 0.05 cc was injected into the yolks of fertile eggs on the 5th day of incubation. One or 2 of these eggs were analysed daily during the remaining 15 days of incubation to determine the location and chemical combination of their P³².

The analysis consisted of first separating the embryo, allantoic and amniotic fluid, yolk, albumen, and shell. Because of technical difficulties the allantoic and amniotic fluids were used together and not separated. The fluids were measured with a pipette or a tuberculin syringe and estimated to the nearest 0.5 cc. Greater accuracy in determination of these fluid volumes was not possible because of some mixing of the fluids during separation. The embryo and shell were weighed. Second, the radioactivity of these various components was measured with a Geiger counter. In order to avoid any errors due to selfabsorption, the embryos were completely ashed before measurement; the shells were dissolved in HCl and an aliquot of this solution was used for measurement. Third, in the eggs from the 6th to the 14th day of incubation each of the three fluid components was divided into ether extract, trichloracetic acid precipitate, and water soluble fraction after precipitation with trichloracetic acid. This was done by mixing 2 cc of the fluid sample with 8 cc of H_2O and extracting with ether for 15 minutes. To the H_2O soluble fraction was added 5 cc of 20% trichloracetic acid. This was filtered. After evaporation the activity of each of these fractions was measured.

Results. Complete results are listed on the accompanying table. Within 24 hours after

injection into the yolk, ⅔ of the P³² had left the yolk and was found principally in the allantois and amnion. After the first day, the balance of labeled phosphate in the yolk remained constant for 4 or 5 days and then gradually diminished in amount until at hatching time about 5% remained in the yolk. During this period the volume of yolk had diminished to about half of its original volume.

Within 24 hours after injection, more than ½ of the P³² was in the allantoic and amniotic fluids. Subsequently, the amount of radioisotope in these fluids decreased in proportion to the uptake by the embryo, until at hatching time they contained only 3-4%. The volume of these fluids was constant until the last 4 days of incubation when it decreased rapidly.

The embryo's content of P³² increased nearly in proportion to its gain in weight. Shortly after injection its concentration was about 1.5 $\mu c/g$ of embryo, and this ratio was maintained until the last 6 days when the concentration was closer to one $\mu c/g$ of embryo. At hatching time the embryo contained about 90% of the P³² and made up a little more than ½ the total weight of the egg.

The albumen never contained more than 3% of the injected radio-isotope. As incubation continued, the drop in P³² more or less paralleled the decrease in volume of albumen.

The shell and its membrane contained only 1% of the P³² throughout incubation. This small amount of activity lies within the experimental error because of the obvious difficulty of separating shell from all fluid contents.

Thus it is apparent that within 24 hours following injection, a shift of P³² from yolk to allantois and amnion takes place. As the embryo grows, its rate of uptake of the phosphate from allantois and amnion is much greater than from the yolk.

In addition to the above analysis we determined for each fluid constituent the amount of P³² contained in the H_2O soluble fraction, the ether soluble fraction and the protein precipitate. The distribution of P³² within

1 Needham, J., *Chemical Embryology,* Vol. II, p. 1199, Cambridge University Press, London, 1931.

TABLE I.
Distribution of P[32] in Incubated Eggs.
(Approximately 5 mg KH$_2$PO$_4$ containing 20μc P[32] injected into yolk 5th day of incubation.)

| Age of egg | Embryo | | Allantois and amnion | | Yolk | | Albumen | | Shell and membrane. |
	Wt in g	% of total activity	Volume in cc	% of total activity	Volume in cc	% of total activity	Volume or wt	% of total activity	% of total activity
6	0.4	3	13.5	64	20	29	17 cc	2	2
7	0.8	6	14	52	20	37	14 ,,	2	1
8	1.4	10	13	53	22	36	12 ,,	1	1
9	2.2	18	15	50	21	29	11 ,,	3	1
10	3.1	28	11	29	26	39	10 ,,	3	1
11									
12	5.8	58	14	21	14	19	8 ,,	1	1
13	8.1	65	10.5	12	16	19	11.5 ,,	3	1
14	9.3	71	13	12	20	17	7 ,,	.7	1
15	13.3	73	13.5	13	14	12	5 ,,	.8	1
16	16.9	73	11	16	14	7	3 ,,	.6	1
17	18.1	76	6.5	17	14	7	1 g		.8
18									
19	25.5	93	3	3	14	4	1 ,,	.3	.3
20	27.4	90	7	4	9	5	.5 ,,	.4	.6

these various fractions was relatively constant throughout our period of observation from the 6th to the 14th day of incubation. Ninety per cent of the P[32] in the allantoic and amniotic fluids was found in the H$_2$O soluble fraction, 9.4% in the trichloracetic acid precipitate and 0.6% in the ether soluble extract.

In the yolk nearly all of the activity was in the H$_2$O soluble fraction, only 1.1% was found in the ether extract.

In the albumen 80% of activity was found in H$_2$O soluble fraction and 20% in trichloracetic acid precipitate.

Summary. The radioactive isotope P[32] was used as a tracer to follow the course of phosphorus combined as KH$_2$PO$_4$ and injected into the yolks of incubating eggs. The distribution of P[32] within the various components of the eggs was determined daily throughout incubation. The distribution in ether soluble, water soluble, and trichloracetic acid precipitable fractions was determined for the fluid components of the eggs from the 6th to the 14th day of incubation. From the 6th day of incubation to the day of hatching, the P[32] in the embryo increased from 3% to 90%.

16148

Changes in Plasma Inorganic Phosphorus of Dogs Under Postural Restraint.*

JOHN G. CONIGLIO.† (Introduced by C. S. Robinson.)

Form the Department of Biochemistry, Vanderbilt University, Schools of Medicine and Nursing, Nashville, Tenn.

During the course of some routine experi-

ments the observation was made that, in unanesthetized dogs restrained in the supine position, the inorganic phosphorus concentration of whole blood and of plasma decreased to a low value of 30-60% of the original amount in 2 hours, after which it increased, in some instances attaining a level about

TABLE I.
Average Plasma Inorganic Phosphorus Values in Dogs Restrained in the Supine Position.

Duration of restraint (min.)	Dog 1 (4 exp.)			Mean of 4 dogs (17 exp.)		Mean of all samples		
	M	S	A	M	A	M	S	A
0-9	4.36	0.34	100.	4.05	100.	4.07	1.10	100.
10-29	3.51	0.30	81.	3.52	87.	3.45	0.94	85.
30-59	2.38	0.35	55.	3.45	85.	2.58	0.62	63.
60-119	2.26	0.55	52.	2.23	61.	2.22	0.80	55.
120-239	2.55	0.94	58.	2.46	67.	2.23	1.20	55.

M   Mean values in mg %.

S   Standard deviation; S equals $\sqrt{\dfrac{\Sigma(X-M)^2}{n-1}}$

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equal to the original value.

Blood phosphate changes in normal animals have frequently been observed upon administration of substances such as insulin or epinephrine[1,2] or upon the ingestion of glucose,[3] but we have found in the literature no report of changes in blood phosphate in animals which, other than being restrained on their backs, were allowed to remain under normal conditions.

In view of these facts and since the phosphate changes we had observed were so marked and consistent, we investigated further the occurrence of this phenomenon under two different conditions of postural restraint: first, that in which the animals were tied in the supine position for 2 to 4 hours and second, that in which they stood in a Pavlov stock for the same period of time.

Methods. In the first group of experiments blood samples were drawn from the femoral vein immediately after the dogs were laid on the table and again at 30, 60, 120, and 240 minutes later. In the second group the animals were placed in a Pavlov stock after a "control" blood sample was taken.

Immediately after being drawn the blood was delivered into cold centrifuge tubes containing dried sodium oxalate and surrounded by crushed ice, centrifuged, and the plasma kept chilled until the inorganic phosphorus analysis was completed.

Inorganic phosphorus was determined by the method of Lowry and Lopez[4] on the ice-cold trichloracetic acid filtrate of the chilled plasma. Total phosphorus, acid soluble phosphorus, and lipid phosphorus, (after extraction with alcohol-ether mixture) all were evaluated after sulfuric-nitric acid digestion (2.5 ml of 5 N sulfuric acid and 3 drops of concentrated nitric acid) by addition of 2 ml of 2.5% ammonium molybdate solution and 3 ml of 1% fresh ascorbic acid solution. Exactly 20 minutes after addition of the ascorbic acid the developed color was measured in a Klett-Summerson photoelectric colorimeter, using filter No. 66. Blanks and standards were always done parallel to unknowns and duplicate samples were analyzed whenever possible.

Plasma glucose was estimated by a modification of the Somogyi method reported by Nelson.[5]

Results and Discussion. Marked decreases in plasma inorganic phosphate averaging 52% of the initial level were observed in all but one of a total of 17 experiments done on 4 dogs in the first group. Table I shows the mean values of all 17 experiments as well as average results of 4 experiments done on Dog 1 as a representative example. The mean of

[1] Perlzweig, W. A., Latham, Emily, and Keefer, C. S., PROC. SOC. EXP. BIOL. AND MED., 1923, 21, 33.

[2] Harrop, George A., Jr., and Benedict, E. M., ibid., 1923, 20, 430.

[3] Harrop, George A., Jr., and Benedict, E. M., J. Biol. Chem., 1924, 59, 683.

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16148

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Methods. In the first group of experiments blood samples were drawn from the femoral vein immediately after the dogs were laid on the table and again at 30, 60, 120, and 240 minutes later. In the second group the animals were placed in a Pavlov stock after a "control" blood sample was taken.

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[4] Lowry, Oliver H., and Lopez, Jeanne A., ibid., 1946, 162, 421.

[5] Nelson, Norton, ibid., 1944, 153, 375.

TABLE II.
Plasma Phosphorus Fractions and Glucose Content, Dog 4, Exp. 17.

Duration of restraint (min.)	Phosphorus (mg %)				Glucose (mg %)	% of initial conc.	
	TP	LP	TASP	IP		IP	Glucose
0⁺	14.9	11.1	3.28	3.04	98.4		
0-9	14.5	11.3	3.34	3.18	107.	100.	100.
10-29	—		—	—	—	—	—
30-59	13.8	11.3	2.76	2.68	107.	84.	100.
60-119	12.5	11.1	0.93	0.97	158.	31.	151.
120-239	12.3	11.1	1.24	1.04	158.	33.	151.

* This blood taken 60 minutes prior to restraining the dog.
TP   Total Phosphorus.
LP   Lipid Phosphorus.
TASP   Total Acid Soluble Phosphorus.
IP   Inorganic Phosphorus.

TABLE III.
Plasma Inorganic Phosphorus Concentration of Dogs Restrained in a Stock.

Duration of restraint (min.)	Inorganic Phosphorus (mg %)		% of initial conc.
	Dog 3	Mean of 4 dogs	
0	4.76	4.64	100.
1-29	4.28	4.08	88.
30-59	4.28	3.79	82.
60-119	4.28	4.04	87.
120-239	4.48	3.98	86.

all the samples, also included in this table, indicates significant decreases through the 60-119-minute period, after which the concentration remained constant or increased slightly in most of the dogs.

In 8 of the 17 experiments performed with the dogs restrained in the supine position a complete analysis of the plasma phosphorus fractions was done, and in 3 of these the plasma glucose levels were also followed. A complete analysis of one of these three experiments is presented in Table II. In this experiment the dog was allowed to remain unrestrained on the floor for about one hour and then tied in the supine position for 2 to 4 hours. The dog was quiet while on the floor but showed signs of unrest soon after being confined. The irritability increased markedly after the 60-minute period and continued until the animal was set free. In all 8 experiments the acid soluble and total phosphorus paralleled the changes in the inorganic fraction, while lipid phosphorus remained quite constant during an entire experiment. In general, increases in plasma glucose were observed at about the same time or immediately after decreases in plasma inorganic phosphorus were noted.

Our animals, although trained to lie quietly on their backs, at times exhibited signs of emotional disturbances and fatigue. A study of the decreases in the obviously excited animals and in the apparently quiet ones revealed an average low value of 40% of the original concentration in the former group compared to 69% for the latter. This difference, when studied statistically, is not significant, the probability at the 60-119-minute period being 17%. Analysis of a larger number of experiments might show a more significant difference between the 2 groups.

The supine position is not a natural one for the dog and restraint in such a position might possibly be the cause of physiological changes in the animal, either directly because of the peculiar posture or indirectly because of aroused emotional status due to fatigue. In 4 experiments dogs restrained in a standing position in a stock did not offer any resistance or become excited to any noticeable

extent. In these experiments, the results of which are presented in Table III, there was a slight decrease in plasma inorganic phosphate, the greatest decrease occurring at the 30-59-minute period and amounting to 13% of the initial value.

It is not possible from the data obtained to derive any conclusions concerning the exact cause of these changes occurring in the plasma phosphorus concentration of restrained dogs. The principal factors appear to be those of posture and of emotional disturbance. This paper does not attempt to establish the relative contribution of each of these factors to the phosphorus decrease; its purpose is to report the observation of this phenomenon so as to bring it to the attention of those in-vestigators whose experiments may be affected by its occurrence.

Summary. 1. A decrease in the plasma inorganic phosphate concentration amounting to 30-60% of the initial concentration was observed in dogs restrained in the supine position. This change was also evident in the total acid soluble and the total phosphorus fractions, while the lipid phosphorus fraction remained constant during an entire experiment.

2. The decrease in inorganic phosphate was accompanied by an increase in plasma glucose.

3. Dogs restrained on their backs exhibited a greater decrease than those restrained in a stock.

16149

Effect of Salicylate and Tripelennamine Hydrochloride (Pyribenzamine) on the Arthus Reaction and on Bacterial Allergic Reactions.[*]

EDWARD E. FISCHEL. (Introduced by R. F. Loeb.)

From the Departments of Medicine and Bacteriology, Columbia University College of Physicians and Surgeons, and the Edward Daniels Faulkner Arthritis Clinic of the Presbyterian Hospital, New York City

The tissue damage that results from the union of certain antigens with their corresponding antibodies may be prevented by non-specific agents.[1,2] In the case of the positive tuberculin reaction, cachexia and fever appear to inhibit the expected reactions.[2] As yet, however, little attention has been directed to the inhibition of necrotizing allergic reactions by therapeutic agents. The more transient reactions of classical anaphylaxis are found to be prevented effectively by the Mosnier compounds which have striking anti-histamine properties.[3,4] Urticarial reactions to specific allergens or to histamine can be inhibited by many of these synthetic compounds, of which the most widely used in this country are diphenhydramine hydrochloride (Benadryl) and tripelennamine hydrochloride (Pyribenzamine). Clinically, hay-fever and analogous reactions have been benefitted by these agents.[4] These substances, however, have not been demonstrated to be beneficial in the "bacterial" type of allergic response, or in the local Arthus reaction, although these allergies may be important in the pathogenesis of many diseases. Reubi[5] reported that 2-(N-phenyl-N-benzyl-aminoethyl)-imidazoline (antistine) prevented the occurrence of the kidney damage that usually results when duck anti-kidney serum is in-

[*] This work was done during the tenure of a Life Insurance Medical Research Fellowship.

[1] Mitchell, A. G., Wherry, W. B., Eddy, B., and Stevenson, F. E., *A. J. Dis. Child.*, 1928, 36, 720.

[2] Gay, F. P., and Associates, *Agents of Disease and Host Resistance*, Springfield, Charles C. Thomas, 1935.

[3] Feinberg, S. M., *J. A. M. A.*, 1946, 132, 703.

[4] Friedlander, S., *Am. J. Med.*, 1946, 1, 174.

[5] Reubi, F., *Helvetia Medica Acta*, 1946, 13, Supp. XVIII.

TABLE II.
Plasma Phosphorus Fractions and Glucose Content, Dog 4, Exp. 17.

Duration of restraint (min.)	Phosphorus (mg %)				Glucose (mg %)	% of initial conc.	
	TP	LP	TASP	IP		IP	Glucose
0*	14.9	11.1	3.28	3.04	98.4		
0-9	14.5	11.3	3.34	3.18	107.	100.	100.
10-29	—	—	—	—	—	—	—
30-59	13.8	11.3	2.76	2.68	107.	84.	100.
60-119	12.5	11.1	0.93	0.97	158.	31.	151.
120-239	12.3	11.1	1.24	1.04	158.	33.	151.

* This blood taken 60 minutes prior to restraining the dog.
TP   Total Phosphorus.
LP   Lipid Phosphorus.
TASP   Total Acid Soluble Phosphorus.
IP   Inorganic Phosphorus.

TABLE III.
Plasma Inorganic Phosphorus Concentration of Dogs Restrained in a Stock.

Duration of restraint (min.)	Inorganic Phosphorus (mg %)		% of initial conc.
	Dog 3	Mean of 4 dogs	
0	4.76	4.64	100.
1-29	4.28	4.08	88.
30-59	4.28	3.79	82.
60-119	4.28	4.04	87.
120-239	4.48	3.98	86.

all the samples, also included in this table, indicates significant decreases through the 60-119-minute period, after which the concentration remained constant or increased slightly in most of the dogs.

In 8 of the 17 experiments performed with the dogs restrained in the supine position a complete analysis of the plasma phosphorus fractions was done, and in 3 of these the plasma glucose levels were also followed. A complete analysis of one of these three experiments is presented in Table II. In this experiment the dog was allowed to remain unrestrained on the floor for about one hour and then tied in the supine position for 2 to 4 hours. The dog was quiet while on the floor but showed signs of unrest soon after being confined. The irritability increased markedly after the 60-minute period and continued until the animal was set free. In all 8 experiments the acid soluble and total phosphorus paralleled the changes in the inorganic fraction, while lipid phosphorus remained quite constant during an entire experiment. In general, increases in plasma glucose were observed at about the same time or immediately after decreases in plasma inorganic phosphorus were noted.

Our animals, although trained to lie quietly on their backs, at times exhibited signs of emotional disturbances and fatigue. A study of the decreases in the obviously excited animals and in the apparently quiet ones revealed an average low value of 40% of the original concentration in the former group compared to 69% for the latter. This difference, when studied statistically, is not significant, the probability at the 60-119-minute period being 17%. Analysis of a larger number of experiments might show a more significant difference between the 2 groups.

The supine position is not a natural one for the dog and restraint in such a position might possibly be the cause of physiological changes in the animal, either directly because of the peculiar posture or indirectly because of aroused emotional status due to fatigue. In 4 experiments dogs restrained in a standing position in a stock did not offer any resistance or become excited to any noticeable

extent. In these experiments, the results of which are presented in Table III, there was a slight decrease in plasma inorganic phosphate, the greatest decrease occurring at the 30-59-minute period and amounting to 13% of the initial value.

It is not possible from the data obtained to derive any conclusions concerning the exact cause of these changes occurring in the plasma phosphorus concentration of restrained dogs. The principal factors appear to be those of posture and of emotional disturbance. This paper does not attempt to establish the relative contribution of each of these factors to the phosphorus decrease; its purpose is to report the observation of this phenomenon so as to bring it to the attention of those in-vestigators whose experiments may be affected by its occurrence.

Summary. 1. A decrease in the plasma inorganic phosphate concentration amounting to 30-60% of the initial concentration was observed in dogs restrained in the supine position. This change was also evident in the total acid soluble and the total phosphorus fractions, while the lipid phosphorus fraction remained constant during an entire experiment.

2. The decrease in inorganic phosphate was accompanied by an increase in plasma glucose.

3. Dogs restrained on their backs exhibited a greater decrease than those restrained in a stock.

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Effect of Salicylate and Tripelennamine Hydrochloride (Pyribenzamine) on the Arthus Reaction and on Bacterial Allergic Reactions.*

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The tissue damage that results from the union of certain antigens with their corresponding antibodies may be prevented by non-specific agents.[1,2] In the case of the positive tuberculin reaction, cachexia and fever appear to inhibit the expected reactions.[2] As yet, however, little attention has been directed to the inhibition of necrotizing allergic reactions by therapeutic agents. The more transient reactions of classical anaphylaxis are found to be prevented effectively by the Mosnier compounds which have striking anti-histamine properties.[3,4] Urticarial reactions to specific allergens or to histamine can be inhibited by many of these synthetic compounds, of which the most widely used in this country are diphenhydramine hydrochloride (Benadryl) and tripelennamine hydrochloride (Pyribenzamine). Clinically, hay-fever and analogous reactions have been benefitted by these agents.[4] These substances, however, have not been demonstrated to be beneficial in the "bacterial" type of allergic response, or in the local Arthus reaction, although these allergies may be important in the pathogenesis of many diseases. Reubi[5] reported that 2-(N-phenyl-N-benzyl-aminoethyl)-imidazoline (antistine) prevented the occurrence of the kidney damage that usually results when duck anti-kidney serum is in-

* This work was done during the tenure of a Life Insurance Medical Research Fellowship.

[1] Mitchell, A. G., Wherry, W. B., Eddy, B., and Stevenson, F. E., *A. J. Dis. Child.*, 1928, 36, 720.

[2] Gay, F. P., and Associates, *Agents of Disease and Host Resistance*, Springfield, Charles C. Thomas, 1935.

[3] Feinberg, S. M., *J. A. M. A.*, 1946, 132, 703.

[4] Friedlander, S., *Am. J. Med.*, 1946, 1, 174.

[5] Reubi, F., *Helvetia Medica Acta*, 1946, 13, Supp. XVIII.

jected into rabbits. Attempts to use similar compounds such as diphenhydramine or tripelennamine to inhibit the tuberculin reaction in animals were unsuccessful.[6] Likewise the use of these drugs in two cases of serum sickness and 3 of rheumatic fever has been disappointing in our experience.

Among the agents said to inhibit the more severe allergic tissue reactions, salicylates have been frequently mentioned. Derick, et al.[7] used aspirin to prevent the appearance of some of the manifestations of serum sickness. Several workers have presented data which led them to believe that salicylates inhibited antibody formation.[7-10] Coburn demonstrated that concentrations of sodium salicylate larger than those usually obtained clinically inhibited the union of antigen and antibody in vitro,[11] and on this basis used salicylates prophylactically to prevent recurrences of rheumatic fever following streptococcus infections.[12]

It appeared of interest to study whether, in the presence of known quantities of antibody and antigen,[16] salicylate or tripelennamine could inhibit the Arthus reaction induced passively in rabbits. The "bacterial" type of allergic reaction in humans actively sensitive to tuberculin or to streptococcal nucleoprotein was also studied.

The Arthus Reaction. The desirability of inducing allergic reactions with known amounts of a single pure antigen and its corresponding antibody is obvious from the data of Kabat.[13] He and his coworkers[14-16]

have presented the techniques for producing anaphylaxis and the Arthus reaction with measured amounts of antigen and antibody. The latter technique was employed in this study.

Albino rabbits (about 2200 g) were injected intracutaneously in single or multiple sites with dilutions of rabbit anti-chicken egg albumin serum containing amounts of antibody nitrogen known to give reactions of minimal and maximal severity.[16] The serum was prepared and analyzed by Dr. E. A. Kabat and kindly given for this study. Controls were injected in equal number simultaneously but were not given drug therapy. After a half hour, known amounts of a solution of 4 times recrystallized egg albumin were injected directly into the sites. The reactions were read at 6, 12, 24 and 48 hours, and those of the untreated animals were compared with groups receiving salicylate or tripelennamine. In some instances biopsies were taken of the involved areas for histological comparison. The intensity of the reactions was graded as previously described:[16] $0 =$ no reaction; $\pm =$ transitory erythema lasting 5 to 8 hours, and absent at 24 hours; $+ =$ erythema lasting 24 hours and measuring up to 1.5 cm in greatest diameter at that time; $++$ erythema 1.5-2.5 cm at 24 hours persisting for one to 2 days, with slight edema and occasional slight brownish discoloration after 24 hours; $+++$ 2-3.5 cm erythema with moderate edema and slight brownish discoloration persisting 2 to 3 days; $++++$ more than 2.5 cm of erythema, marked edema and moderate or marked brownish discoloration with changes lasting 3 to 5 days. Rabbits of the treated groups were given the medication every 4 hours on the day before, the day of, and occasionally on the day following the induction of the Arthus reaction, although by this time the reactions had reached their height and readings were conclusive. Sodium salicylate was given intravenously in doses of 2 to 5 ml of a 10%

6 Birkeland, M., and Kornfeld, L., *J. Bact.,* 1947, 54, 82.

7 Derick, C. L., Hitchcock, C. H., and Swift, H. F., *J. Clin. Invest.,* 1928, 5, 427.

8 Swift, H. F., *J. Exp. Med.,* 1922, 36, 735.

9 Homburger, F., Proc. Soc. Exp. Biol. and Med., 1946, 61, 101.

10 Jager, B. V., and Nickerson, M., *Am. J. Med.,* 1947, 3, 408.

11 Coburn, A. F., and Kapp, E. M., *J. Exp. Med.,* 1943, 77, 173.

12 Coburn, A. F., and Moore, L. V., *J. Pediat.,* 1942, 21, 180.

13 Kabat, E. A., *Am. J. Med.,* 1947, 3, 535.

14 Kabat, E. A., and Landow, H., *J. Immunol.,* 1942, 44, 69.

15 Kabat, E. A., and Boldt, M. H., *J. Immunol.,* 1944, 48, 181.

16 Fischel, E. E., and Kabat, E. A., *J. Immunol.,* 1947, 55, 337.

TABLE I.
Effect of Salicylate and Tripelennamine on Severity of the Arthus Reaction Induced Passively with Known Amounts of Anti-egg Albumin and Crystalline Egg Albumin. Number of Reactions of Different Severity Related to Total Reactions in Each Group.

Antibody nitrogen (mg)	Treatment	Severity of reaction with 0.13 mg crystalline egg albumin nitrogen				
		++++	+++	++	+	0
.53	Control	3/3				
	Salicylate	2/3	1/3			
.22	Control	3/16	9/16	4/16		
	Salicylate	1/8	5/8	2/8		
	Tripelennamine		4/10	6/10		
.10	Control			1/2	1/2	
	Tripelennamine				2/3	1/3
.05	Control			4/7	3/7	
	Salicylate			4/7	2/7	1/7
	Tripelennamine			4/5	1/5	
.025	Control				2/2	
	Tripelennamine				1/3	2/3

solution (0.2 to 0.5 g). In a few instances serum salicylate levels were determined.[17] A value of 1224 γ per ml was found a few minutes after one injection of 0.4 g and 311 γ per ml after 4 hours. Tripelennamine hydrochloride (Pyribenzamine) was administered as an 0.5% solution in doses from 20 mg intramuscularly to 5 mg intravenously on the day before and at 4-hour intervals on the day of induction of the Arthus reaction. Ten mg intravenously caused transient convulsions in some rabbits.

The results of the systemic administration of salicylate or Pyribenzamine on the severity of the passive Arthus reaction are presented in Table I. It is apparent that the severity of the reaction was not appreciably altered when the higher concentrations of antibody were used, or with quantities normally giving a minimal reaction. The readings tabulated are those at the end of 24 hours, in conformity with our previous experience, and with the generally accepted picture of the Arthus phenomenon. However, during the first 6 or 8 hours a difference between the control and salicylate treated groups was noted which does not lend itself to adequate measurement. Usually the control and tripelennamine treated groups had a thicker and slightly wider area of edematous skin than did the

salicylate treated group. The reaction at 24 hours more closely approximates the tissue damage associated with the classical Arthus phenomenon and apparently the end reaction is not influenced by the moderate difference in edema at the end of 6 hours.

Bacterial Allergic Reaction. "Bacterial" allergic reactions have been distinguished from the Arthus reaction chiefly because it is not possible to transfer the allergy to bacterial products which results after infection with serum of sensitized individuals.[18] Chase[19] showed that sensitivity of the tuberculin type may be passively transferred with cellular exudates from sensitized animals. The mechanism of this cellular transfer of sensitivity is not yet clearly understood. In these studies it was necessary to determine the initial degree of sensitivity and then subject the same individual to the salicylate or tripelennamine and ascertain the effect on the reaction.

Subjects were adults on the wards of the Presbyterian Hospital recovering from vari-

17 Brodie, B. B., Undenfriend, S., and Coburn, A. F., J. Pharm. and Exp. Therap., 1944, 80, 114.

18 Rich, A. R., The Pathogenesis of Tuberculosis, Springfield, Charles C. Thomas, 1941.

19 Chase, M. W., PROC. SOC. EXP. BIOL. AND MED., 1945, 59, 134.

TABLE II.

Effect of Salicylate and Tripelennamine on Intracutaneous Skin Tests in Individuals Sensitive to Streptococcal Nucleoprotein Fraction C18K.

Subject	Area of erythema			
	Control (inches²)	Salicylate (inches²)	Salicylate level (γ/ml)	Tripelennamine (inches²)
R.A.	.15	.20	321	.23
E.C.	.33	.31	548	.53
L.C.	.30	.50	352	.39
B.C.	.11	.19	534 (toxic)	.17
B.D.	.30	.42		.28
H.H.	.35	.32	278	.58
Y.M.	.05	.14	338	
M.R.	.16	.26		.25
A.S.	.50	.58		.60
P.W.	.40	.10	507 (toxic)	.60
D.	.27	.40		
X.C.	.19	.05		
Y.C.	.07	.12		
I.W.	—	.37		.50

ous diseases, medical students, and children convalescing from rheumatic fever at the Pelham Home for Children.[†] Intracutaneous skin tests were done on comparable areas of the forearm but individual sites were used only once. The purified protein derivative (PPD) of tuberculin[20] and a nucleoprotein fraction C18K prepared from the hemolytic streptococcus by Heidelberger and Kendall[21] and kindly given for this study by Dr. Heidelberger, were used as antigens. The PPD was used in one of the 2 customary strengths, 0.00002 mg in 0.1 ml and 0.005 mg in 0.1 ml. The C18K was diluted from an analyzed (Kjeldahl) stock solution so that 0.1 ml contained 0.0001 mg nitrogen. Readings were made as frequently as possible, every 6 or 12 hours after injection. The C18K skin test appeared to be at its maximum at 24 hours in all cases except the most severe reaction which went on to become hemorrhagic at 48 hours. The other cases showed well demarcated areas of erythema and edema or induration, occasionally surrounded by a zone of erythema alone. In instances where the zone was present, the areas of both ovals were determined, but only the outer one is listed in Table II. Skin reactions were recorded by cellophane tracings of the lesions and an appraisal of the degree of erythema and induration was made to compare further the reactions in the same individual. Medication was given orally, either a week before or a week after a control test was performed. Salicylate was given as enteric coated aspirin or sodium salicylate in doses of 0.6 to 1.2 g every 4 hours, night and day, giving salicylate levels from 153 γ/ml to 534 γ/ml in a representative group of subjects. Tripelennamine (Pyribenzamine) was given in doses of 25 to 50 mg 3 times a day for a few days before and during the skin reacting period.

Tests were done 2 or 3 times in the same individual, one time without any medication and the other one or 2 times at weekly intervals with salicylate or tripelennamine administered. The results of the C18K skin tests are seen in Table II, which lists the area of the cellophane tracings of the skin lesions as determined by planimeter. Other characteristics of the lesions such as degree of erythema and induration are not listed since they did not vary appreciably from test to test in the same individual.

Thirteen subjects were tested with C18K with and without salicylate administration.

† We are grateful to Miss Mary C. Kelly and the Pelham Home for Children for their cooperation.

20 Seibert, F. B., Aronson, J. D., Reichel, J., Clark, L. T., and Long, E. R., Am. Rev. Tuberc., 1934, 30, Supp.

21 Heidelberger, M., and Kendall, F. E., J. Exp. Med., 1931, 54, 515.

Of these only 2 showed diminution of the area of the skin reaction of more than 0.1 square inch during aspirin administration, one of these had marked signs of salicylism and a level of 507 γ/ml. Seven individuals had no difference in area with and without salicylates and 4 had increases of more than 0.1 square inch despite comparable doses of salicylate. With tripelennamine, the C18K reaction was approximately the same as the control reaction in 5 individuals and increased 0.1 square inch or more in 4 individuals. A similar degree of variation is present when the reactions with salicylate are compared with those during tripelennamine administration. Four individuals did not show any change, 2 had lesions which diminished by more than 0.1 square inch and four showed areas which increased by more than 0.1 square inch. With the tuberculin test, 22 subjects were given aspirin; 14 showed no appreciable change from their control skin test, 5 showed a more severe reaction, and three had a less severe reaction.

Discussion. The Arthus reaction is a manifestation of the union of certain antigens with antibodies, and its severity can be related to the amount of antibody uniting with the antigen.[16,22,23,24] Since the severity of the Arthus reaction was not altered appreciably it does not appear that the drugs employed inhibit the union of antigen with antibody *in vivo*. However, the moderately diminished edema at the end of 6 hours in the salicylate treated group may be of interest in view of the demonstrated inhibition by salicylate of spreading due to hyaluronidase.[25]

Tripelennamine has been shown to exhibit a significant antihistamine action.[3,4] Since it did not lessen the severity of either lesion studied, it appears that histamine does not play a significant role in the pathogenesis of necrotizing allergic reactions.

Summary. A study was made of the effect of salicylate and of a synthetic antihistamine compound, tripelennamine hydrochloride, on necrotizing allergic reactions of the Arthus and "bacterial" types. The Arthus reaction was induced passively in rabbits by quantitative methods. "Bacterial" sensitivity to a streptococcus nucleoprotein fraction and to tuberculin was observed in human subjects. Neither type of reaction appeared to be altered by the administration of salicylate or tripelennamine. However, in the early development of the Arthus phenomenon, salicylate treated animals exhibited less edema at the site of the lesion.

[22] Opie, E. L., *J. Immunol.*, 1924, **9**, 231.

[23] Culbertson, J. T., *J. Immunol.*, 1935, **29**, 29.

[24] Cannon, P. R., and Marshall, C. E., *J. Immunol.*, 1941, **40**, 127.

[25] Guerra, F., *J. Pharm. and Exp. Therap.*, 1946, **87**, 193.

16150

Further Observations on the Cultivation of Strains of Poliomyelitis Virus in Developing Eggs.[*]

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In an earlier paper[1] we reported the successful cultivation of the murine SK strain in developing eggs. In the present paper we wish to summarize observations made since then on this and other strains.

Murine SK Strain. The SK strain has now

[1] Schultz, E. W., and Enright, J. B., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 8.

been carried through 30 passages in fertile eggs. Its ID_{50} for mice in 15th, 21st, 24th and 30th egg passages was 10^{-7}, based on titrations of Chamberland L_3 filtrates of 1% suspensions of whole embryo tissues in half-strength Martin's peptone solution, the filtrates themselves showing a titer of 10^{-5}. This titer was reached by the 4th or 5th day. The average incidence of embryo deaths between the 2nd and 6th days was 79% in inoculated eggs incubated at 35°C, and 46% in eggs incubated at 37.5°C. The virus content of the embryo tissue did not appear to be influenced by the temperature at which the inoculated eggs were incubated.

Up to the 18th passage, the material used to inoculate the next group of eggs consisted of the heads and necks only of the embryos from the preceding passage. After the 18th passage, the entire embryo was used in preparing the passage material. Nine-day eggs were employed in making the passages. All were inoculated by the chorioallantoic route. The inoculum in all passages consisted of 0.1 ml of Chamberland L_3 filtrates of 1% suspensions of the chick embryo tissue in half-strength Martin's peptone solution. Twelve supplementary passages were made by the yolk sac route. In the latter, the passage material consisted of filtrates prepared from suspensions of abdominal viscera only. This series showed about the same virus content at the end of the 12th passage as the series carried with material from the heads and necks only. In the series inoculated by the chorioallantoic route, the allantoic fluid, unfiltered, showed an ID_{50} of 10^{-5} in the 9th passage, the chorioallantois itself an ID_{50} of 10^{-4} in the 5th passage, and the yolk only in the 15th passage titered only 10^{-1}.

Embryos from 5 to 14 days of age seemed to serve equally well for the propagation of the virus. However, in eggs containing 15- and 16-day-old embryos the ID_{50} dropped from the usual level of 10^{-7} to 10^{-4}, and in 17-day-old eggs to zero. With this drop in titer the incidence of embryo deaths also fell off. Attempts to adapt the virus to older embryos failed.

Neutralization tests on material from the 24th egg passage did not show any appreciable change in the antigenic properties of the virus.

Murine MM Strain. This strain, described by Jungeblut and Dalldorf[2] was obtained from Dr. Jungeblut in 1946 in its 107th mouse passage. Jungeblut[3] briefly mentions that this strain had been "carried over five serial passages in fertilized hen's eggs." In a later paper, Brutsaert, Jungeblut and Knox[4] state that the virus could not be recovered after the 5th or 6th serial passage.

We succeeded in carrying the MM strain through 10 egg passages under conditions similar to those employed for the SK strain. The ID_{50} for mice of material from the 10th egg passage was about 10^{-6}, based, as in the preceding series, on serial dilutions of Chamberland L_3 filtrates of 1% suspensions of whole embryo tissue. At 35°C the average incidence of embryo deaths was 75%, based on a total of 52 eggs employed; at 37.5°C this incidence dropped to about 20%.

Murine Lansing or C(M) Strain. Of especial interest have been the results obtained with what appears to be a murine high titer variant of the Lansing strain. This strain prior to its trial in eggs had been passed continuously in mice over a period of more than four years. Just when it first showed itself as a high titer virus we are unable to say since actual titrations were not carried out during most of this period. Up to 1943 it behaved like a typical "low titer" Lansing strain. However, shortly before the present studies were undertaken in 1946, it showed an ID_{50} of 10^{-7} for mice. The possibility that the supposed variant was actually a foreign virus obviously had to be considered. An investigation was therefore undertaken to determine its relationships to Lansing "passage strains" from other laboratories. This study is still in progress, and will be reported later.[5] Its inclusion in this report—as a probable variant of the Lansing strain—seems

2 Jungeblut, C. W., and Dalldorf, G., *Am. J. Pub. Health*, 1943, **33**, 169.

3 Jungeblut, C. W., *J. Exp. Med.*, 1945, **81**, 275.

4 Brutsaert, P., Jungeblut, C. W., and Knox, Alice, *J. Pediat.*, 1946, **29**, 350.

to be justified by the results of serological observations thus far and by the fact that it has produced characteristic flaccid paralysis in individual monkeys when these were inoculated intracranially with mouse brain-cord virus suspensions. In one group of 3 monkeys inoculated with "autolyzed" brain-cord material from recent mouse passage prepared according to Milzer and Byrd,[6] all developed typical paralysis with characteristic cord lesions.

This virus has been carried through 15 egg passages. As in the murine SK series the passages were initiated with a Chamberland L_3 filtrate of a 1% suspension of mouse brain-cord tissue in half-strength Martin's peptone solution. The ID_{50} of this filtrate was 10^{-5}, or not less than 10^{-7} for the unfiltered material. This was seeded to 5 eggs in 0.1 ml amounts, by the chorioallantoic route. All subsequent passages were made as described for the preceding viruses. The virus has been carried through the 15 passages without apparent loss of its ID_{50} for mice. Up to the 5th passage, the material passed consisted of heads and necks only of the preceding embryos. In all subsequent passages filtrates of ground whole embryo tissue were employed. Of a total of 78 eggs inoculated in making these passages, 76% developed dead embryos between the 2nd and 6th days.

While the infectivity for mice of the egg passage material has remained high (10^{-7}), its infectivity for monkeys has proved to be low. An undiluted L_3 filtrate of a 1% suspension of material harvested from the 5th egg passage was inoculated into 4 Rhesus monkeys, each animal by both the intracerebral and intraperitoneal routes. No symptoms were observed until the 3rd week, at which time all 4 of the animals developed a well-defined awkwardness in their movements. None developed a definite paralysis, however. Three of the animals were sacrificed soon after the onset. No well-defined cord lesions were observed in any of them, and only one

showed perivascular cuffing in the brain. The 4th animal recovered without any residual manifestations. A similar filtrate from the 10th egg passage was inoculated, undiluted, into 2 monkeys. Both failed to develop symptoms, despite the fact that the ID_{50} of this particular filtrate, for cotton rats, was 10^{-4} and 10^{-5} for mice (or not less than 10^{-6} and 10^{-7} respectively, for the unfiltered material). Employed in a dilution of 10^{-4}, this filtrate was neutralized by an equal volume of anti-C(M) serum produced in a rabbit (final serum dilution 1:5), but not by antisera against the murine SK strain and the GD VII strain of Theiler's virus when these antisera were employed in similar dilutions. Certain tests carried out with mouse brain-cord suspensions have shown, however, that there is some cross reaction with the murine SK strain.[5]

Theiler's Encephalomyelitis Virus. The GD VII strain of Theiler's virus was carried through 10 egg passages. Success with strains of Theiler's virus had been reported, however, by earlier workers.[7,8,9] The ID_{50} of an L_3 filtrate of material from the 10th passage, prepared from a pool of whole embryos, was 10^{-5}. With this particular virus the average incidence of embryo deaths was only 23%, based on a total of 55 eggs employed. The virus was neutralized by an homologous antiserum produced in a rabbit but not by anti-murine SK serum nor by anti C(M) serum.

Strains Which Failed to Grow in Fertile Eggs. The following strains failed to show growth in developing eggs: (1) the cavian SK strain,[10] obtained from Dr. Claus W. Jungeblut in 1946 as his 74th serial passage in guinea pigs. (2) the original monkey passage SK strain,[11] obtained from Dr. John

[5] Schultz, E. W., and White, S. C., unpublished observations.

[6] Milzer, A., and Byrd, C. L., *Science*, 1947, 105, 70.

[7] Gard, S., *Acta Med. Scand.* (Suppl), 1943, 1943.

[8] Dunham, W. B., and Parker, Sue, *J. Bact.*, 1943, 45, 80.

[9] Riordan, J. T., and Sá-Fleitas, M. J., *Science*, 1946, 103, 449.

[10] Jungeblut, C. W., PROC. SOC. EXP. BIOL. AND MED., 1945, 58, 177.

[11] Trask, J. D., Vignec, A. J., and Paul, J. R., PROC. SOC. EXP. BIOL. AND MED., 1938, 38, 147; 1939, 41, 241; *J. A. M. A.*, 1938, 111, 6.

R. Paul in 1946 in its 22nd passage, (3) a cotton rat passage strain of the Lansing virus, obtained from Dr. Hubert Loring in 1946, (4) the MV strain, and (5) the FW strain isolated by one of us (E.W.S.) from the stools of a poliomyelitis patient in 1941. All 5 of these strains failed to prove infectious for test animals after the 5th or 6th egg passages. Since the L₃ filtrate of the Lansing strain had failed to induce infection in mice, the egg passages in this series were initiated with 0.2 ml of an unfiltered 1% suspension mouse brain-cord tissue in half-strength Martin's peptone solution of the passage material, which did prove infectious for 5 out of 6 cotton rats.

After we had completed this work a paper by Riordan and Sá-Fleitas[12] appeared in which they also report having obtained negative results with certain "monkey pathogenic mouse adapted strains" of poliomyelitis virus.

Summary. Further observations on the cultivation of the murine SK strain of poliomyelitis virus in fertile eggs are reported. This strain has now been carried through 30 passages in eggs. The virus has been found to be widely distributed in the infected embryo. It is easily transmitted from egg to egg by different routes. Embryos from 5 to about 14 days of age seem to serve equally well for the propagation of the virus, but those above 16 days of age fail to support its growth.

The murine MM strain was carried through 10 egg passages; a Stanford mouse passage strain, believed to be a high titer variant of the Lansing strain, tentatively labeled C(M) virus, was carried through 15 egg passages. The GD VII strain of Theiler's mouse encephalomyelitis virus was carried through 10 passages. It caused the lowest incidence of embryo deaths of the three viruses cultivated. Five strains of poliomyelitis virus failed to show growth in eggs.

12 Riordan, J. T., and Sá-Fleitas, M. J., *J. Immunol., Virus Research and Exp. Chemotherapy,* 1947, 56, 263.

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Ability of Diisopropylfluorophosphonate (D.F.P.) to Produce Antidromic Activity in Motor Nerves.

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Masland and Wigton[1] demonstrated antidromic activity in motor nerves, occurring in association with fascicular twitching of the muscles produced by neostigmin (Prostigmin), or by eserin. They concluded that the cholinesterase inhibiting action of these drugs led to an accumulation of acetylcholine, which stimulated the motor nerve at its ending, and initiated antidromic impulses. This work was further extended by Eccles, Katz, and Kuffler.[2] These investigators demonstrated that the antidromic activity observed in the motor nerve sometimes originated in the muscle fibre, and at other times originated in the nerve ending itself.

In view of the development of new anticholinesterase drugs, it was of interest to determine whether the new drugs produce similar antidromic activity. A study similar to that previously reported with neostigmin has therefore been made using diisopropylfluorophosphonate (D.F.P.). The drug was injected intramuscularly or intraperitoneally in cats, in doses of 5 to 10 mg, as a 1% solution in peanut oil. Active fascicular twitching of the muscles was usually observed in about 40 minutes from the time of injection. When the drug was injected intramuscularly,

1 Masland, R. L., and Wigton, R. S., *J. Neurophysiol.,* 1940, 3, 269.

2 Eccles, J. C., Katz, B., and Kuffler, S. W., *J. Neurophysiol.,* 1942, 5, 211.
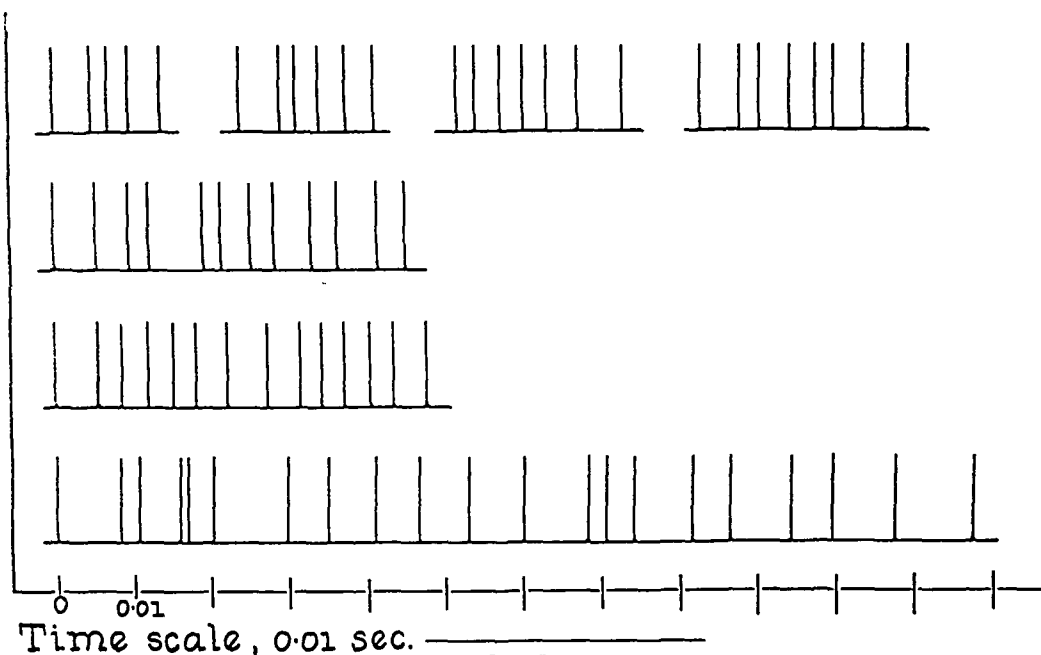
FIG. 1.

Diagram showing the spike frequency and the duration of 7 typical grouped discharges produced by D.F.P. and recorded from an anterior root of the cat. The bursts diagrammed contain from 5 to 21 spikes. Bursts consisting of a single spike, which also occurred, have not been indicated.

the twitching usually appeared first in the limb into which the drug had been injected. When recording electrodes were applied to the motor nerve roots within the spinal canal under these conditions, antidromic activity was recorded. The activity consisted of repeated bursts of nerve impulses, the impulses occurring in groups of 1 to 20, at frequencies up to 200 second during the bursts. The activity appeared to be identical with that previously reported in connection with the administration of neostigmine. (Fig. 1).

These experiments provide further evidence of the similarity of action of eserine, neostigmine, and D.F.P., and support the view that the effect of these drugs on the neuromuscular system is a result of their common action as cholinesterase inhibitors.

16152

Galactose Excretion in Young and Hepatoma Rats Fed Skim Milk Diets.*

P. H. DERSE, C. A. ELVEHJEM, AND E. B. HART.

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Adult rats receiving liquid skim milk ex-

crete as high as 35% of the ingested galactose in the urine. When whole milk or skim milk supplemented with 3 or 4% fat is fed, the urinary loss of galactose is greatly reduced.[1] Fat increases the utilization of galactose by

[1] Schantz, E. J., Elvehjem, C. A., and Hart, E. B., J. Biol. Chem., 1938, 122, 381.

the rat when either lactose or galactose is ingested in milk or synthetic type rations. The percent of the ingested galactose lost in the urine is independent of the actual amount of galactose ingested but is dependent upon the per cent of galactose in the ration.[2,3] The rate of intestinal absorption of galactose varies inversely with the percentage of fat in the diet.[3]

Heretofore, in the galactose excretion studies, adult rats were employed. This report presents results of tests with weanling rats and rats with hepatoma.

Weanling male albino rats of the Sprague-Dawley strain were kept in individual wire mesh metabolism cages. Urine samples were collected under toluene for 24-hour periods. Once or twice weekly the galactose content was determined by using a slight modification of the Shaffer-Hartmann method[4] with a factor of 1.22 to convert the values to galactose. Numerous urine samples were inoculated with *Saccharomyces cerevisiae* to test for fermentable reducing substances and in no case did this cause the amount of reducing substances to decrease.

The diet of the animals was prepared fresh daily by incorporating 10 g of commercial skim milk powder in water to a volume of 100 cc. To each 100 cc were added 12 mg of ferric pyrophosphate (Mallinckrodt N. F. VII), 0.6 mg of $CuSO_4 \cdot 5H_2O$ and 0.6 mg of $MnSO_4 \cdot 4H_2O$. The liquid diet contained 4.65% lactose of 2.45% galactose. The fat soluble vitamins were supplied as a corn oil concentrate, 2 drops per week furnishing 1.568 mg of α-tocopherol, 0.147 mg of 2-Me-1,4-naphthoquinone, 0.35 mg of β-carotene,[†] and 0.0098 mg of calciferol.[‡]

Four duplicate experiments, consisting of 6 rats each, were set up at different intervals. The average weekly galactose excretion values obtained with each group are given in Table I

The studies were repeated on rats with hepatomas,[§] induced by p-dimethylaminoazobenzene or m'-methyl-p-diaminoazobenzene.[5] The hepatomas were ascertained by palpation. The results of these tests are presented in Table II.

Discussion. The urinary excretion of galactose in the weanling rat was lower than that of the adult animal on the skim milk diet. The excretion values (Table I) increased during the fifth and sixth week until the rate of excretion of the adult rat was reached. During the first few days on the liquid diet slight weight losses were observed in each group of rats, but thereafter slow growth occurred. Diarrhea was surprisingly absent in most of the young animals. When present, the excretion value was omitted due to unavoidable contamination of the urine sample. After 3 or 4 weeks on the experimental diet, 55% of the young rats had developed an opacity of the lens. This incidence was surprisingly high. Krewson *et al.*[6] had reported that the lactose content of skim milk alone was not great enough to produce cataracts. Their work differed from this in that their rats were beyond the weanling stage, weighing 80 to 100 g.

Adult hepatoma rats fed the skim milk diet showed excretions of galactose varying from zero to $\frac{1}{5}$ the amount excreted by normal adult rats. A slow increase in excretion was noted in Rats 1 and 5 from Groups 2 and 3 respectively. Autopsy of these 2 animals showed no evidence of hepatoma. The high mortality of the tumor animals tended to obscure the results and prevent the completion of most of the experimental runs.

The lower excretion in the weanling and tumor rats approximates that obtained when 3 or 4% of fat is incorporated in the milk.

2 Geyer, R. P., Boutwell, R. K., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1945, **162**, 251.

3 Nieft, M. L., and Deuel, H. J., Jr., *J. Biol. Chem.*, 1947, **167**, 521.

4 Osgood, E. E., *A Textbook of Laboratory Diagnosis with Clinical Applications for Practitioners and Students*, Philadelphia, 1935, 301.

† 90% β-carotene and 10% α-carotene.

‡ Crystalline irradiated ergosterol.

§ The hepatoma animals were obtained through the courtesy of Dr. C. A. Baumann and coworkers.

5 Rusch, H. P., Baumann, C. A., Miller, J. A., and Kline, B. E., *A. A. A. S. Research Conference on Cancer*, 1944, 267.

6 Krewson, C. F., Schantz, E. J., Elvehjem, C. A., and Hart, E. B., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 573.

TABLE I.
Galactose Excretion by Male Weanling Rats.

| Group | Weekly galactose excretion values* (% of galactose ingested) | | | | | | | | | | No. of animals with lens opacity |
	1	2	3	4	5	6	7	8	9	10	
1	5.69	8.66	8.60	9.7	12.02	—	—	21.7	25.1	—	5
2	7.41	6.46	5.03	3.6	12.20	20.5	20.9	27.7	—	—	2
3	—	6.90	4.45	10.3	11.40	20.1	19.1	32.0	40.0	31.0	3
4	4.61	4.61	8.35	11.56	14.40	16.77	19.8	29.1	—	—	3

* Each group consisted of 6 animals, but contamination due to diarrhea sometimes occurred. Because the contaminated samples were omitted, each figure in the table represents the average urinary galactose excretion of 3 to 6 animals.

TABLE II.
Galactose Excretion by Male Rats with Hepatoma, Fed Skim Milk.

| Group | Rat | Weekly galactose excretion values* (% of galactose ingested) | | | | | | | | | |
		1	2	3	4	5	6	7	8	9	10
I	1	5.05	5.05	Dead							
	2	2.98	3.38	6.49	Dead						
	3	2.98	Dead								
	4	0.0	4.3		3.38	5.37	4.78	2.39			
	5	—	—	5.97	8.96	9.23	Dead				
	6	—	—	0.0	3.78	4.78	2.98				
II	1	5.97	6.75	12.30	17.6	24.6	26.0				
	2	6.73	2.58	Dead							
	3	5.69	5.15	4.50	Dead						
III	1	5.97	—	—	0.0	—	—	3.78	5.97	Dead	
	2	6.97	—	—	0.0	—	—	5.97	0.0	Dead	
	3	9.25	—	—	2.98	—	—	6.75	Dead		
	4	4.55	—	—	1.79	—	—	4.30	2.98	4.08	5.97
	5	3.38	—	—	1.79	—	—	20.01	25.40	31.00	33.40
IV	1	4.80	9.4	Dead							
	2	2.92	Dead								

* Each figure represents the galactose excretion value of one animal.

In the young animal more efficient utilization of galactose may be necessary for the more adequate utilization of the high lactose diet during early life. Evidence that the system involved may be one present in the liver is substantiated by the results obtained in rats with hepatomas.

Kosterlitz[7] and others have shown that an increased amount of glucose is found in the liver during galactose assimilation. Greenstein[8] reports that rat hepatoma possesses an acid phosphatase activity double that of normal liver and an alkaline phosphatase activity 120 times as great. This may explain the decreased galactose excretion in the tumor animal. A similarly increased phosphatase system may occur in the young animal, and induce a greater conversion of galactose-1-phosphate to glucose or glycogen. In the older, more mature animal the lactose intake is smaller and the galactose utilization system must compete to a lesser degree. The fact that the adult liver can convert galactose at a certain rate forms the present basis for the galactose tolerance test as a measure of liver function. This study would seem to indicate that the test may be inadequate in determining dysfunction due to tumor.

The hepatomas were ascertained to be originally present through palpation. However, in 2 observations autopsy failed to show

7 Kosterlitz, H. W., Biochem. J., 1943, 37, 181.
8 Greenstein, J. P., J. Nat. Cancer Inst., 1942, 2, 511.

the rat when either lactose or galactose is ingested in milk or synthetic type rations. The percent of the ingested galactose lost in the urine is independent of the actual amount of galactose ingested but is dependent upon the per cent of galactose in the ration.[2,3] The rate of intestinal absorption of galactose varies inversely with the percentage of fat in the diet.[3]

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6 Krewson, C. F., Schantz, E. J., Elvehjem, C. A., and Hart, E. B., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 573.

GRAPH 1.

500,000 units crystalline penicillin dissolved in 5 cc distilled water injected rapidly into right brachial vein through an 18-gauge needle.

trated solution into the brachial vein were as great as those that could be obtained by introducing the penicillin directly into the right side of the heart. In the animals, in addition to extremely high levels in the aorta immediately following injection there were found unusually elevated peripheral blood levels. No untoward manifestations supervened when one million units of crystalline penicillin, dissolved in 5 cc of distilled water, were injected as rapidly as possible through a large (18 gauge) needle into the brachial vein of a dog weighing 18 kg (55,600 units/kg). A continuous electro-

cardiogram taken before, during and following the injection failed to show any alterations in rate, rhythm, or contour of the complexes.

Following these preliminary experiments, the method was applied to patients. The first patient received one million and the second, 2 million units of crystalline penicillin, dissolved in 5 cc of distilled water. The solutions were injected rapidly into the right antecubital vein through a 15-gauge needle, the injection being completed within 5 seconds. No clinical or electrocardiographic abnormalities were noted.

The levels of penicillin in the aortic blood,

any evidence of liver tumor. Whether co-incidental or not, the influence of the liquid skim milk diet on the resolving of hepatomas and other related disturbances is worthy of investigation.

Conclusions. The urinary excretion of galactose in the young rat is lower than that of the adult animal when a supplemented skim milk diet is fed.

On such a diet, adult rats, with hepatoma induced by azo dyes, excrete a lower percentage of galactose in the urine than do normal adult rats.

16153

A Method for Producing Sustained High Penicillin Levels in the Blood.

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(Introduced by Gregory Shwartzman.)
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Improvement in antibiotic therapy may be expected if high penicillin levels could be maintained in the blood stream and tissues for prolonged periods. The required period of therapy may perhaps thereby be shortened and infections due to exceptionally resistant organisms may more readily be brought under control. Furthermore, high, sustained penicillin blood levels may result in tissue saturation and greater penetration of the drug into foci of infection.[1]

To date, efforts to achieve prolonged levels consisted in the employment of two methods, (a) the use of oil, wax and other menstrua to delay absorption of penicillin introduced into a local depot and (b) the use of drugs capable of delaying the ordinarily rapid excretion of penicillin through the kidneys, *i.e.* para-aminohippuric acid,[2] and carona-mide.[3] Caronamide (4'-carboxyphenylmeth-anesulphonanilide) has been shown by Beyer[4] to have an inhibitory effect on the renal tubular excretion of penicillin. In addition, attempts have been made to obtain high levels by continuous intravenous drip and by the periodic administration of large "booster" doses of the antibiotic. The dose and speed of injection have been limited under the continuous intravenous method because of thrombo-phlebitis.

In this study, we have achieved sustained and unusually high levels of penicillin in the blood stream by the periodic intravenous injection of large doses of crystalline penicillin* when caronamide† was used as an adjuvant to block rapid excretion through the kidney.

Initially it was planned to obtain high intracardiac levels of penicillin by introducing the drug through an intracardiac catheter passed into the right ventricle or pulmonary artery. Preliminary experiments on dogs demonstrated, however, that the levels‡ in the aorta achieved by rapid injection of equally large doses of penicillin in concen-

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* Sodium and calcium salts of crystalline penicillin kindly supplied by Schenley Laboratories, Inc.

† Kindly supplied by Sharp and Dohme, Inc.

‡ The method of penicillin assay employed is a broth tube dilution method using *Staphylococcus aureus* H as the test organism and fresh meat extract broth as the medium. The minimal concentration of the standard penicillin required to inhibit the inoculum of 5 x 10² *Staphylococcus aureus* H cells was 0.02 units per ml. The standard penicillin was obtained from the U. S. Department of Agriculture. All titrations of serum levels were accompanied by and compared with this standard.

1 Gerber, I. E., Shwartzman, G., and Baehr, G., *J. A. M. A.*, 1946, **130**, 761.

2 Beyer, K. H., Flippin, H. F., Verwey, W. F., and Woodward, R., *J. A. M. A.*, 1944, **126**, 1007.

3 Crosson, J. W., Boger, W. P., Shaw, C. C., and Miller, A. K., *J. A. M. A.*, 1947, **134**, 1528.

4 Beyer, K. H., *Science*, 1947, **105**, 94.

TABLE II.
Peripheral Blood Penicillin Levels in Units per ml After the Administration of 10 Million Units of Crystalline Penicillin in 10 Hours.

Time	One million units of crystalline penicillin, dissolved in 5 cc distilled water, rapidly inj. into right antecubital vein, hourly, for 10 hrs			Time, hr	Ten million units of crystalline penicillin, dissolved in one L. physiological saline, administered by slow intravenous drip in 10 hrs	
	Patient 5 Caronamide	6 Caronamide	7 No caronamide		8 Caronamide	9 No caronamide
After 1st injection:						
3 min.	184	184	120	1	27	20
5 ,,	160	120	80	2	96	32
30 ,,	96	88	24	3	96	48
55 ,,	64	64	8	4	104	40
				5	112	48
After 5th inj.:				6	120	40
3 min.	224	228	128	7	136	32
5 ,,	208	176	96	8	128	—
30 ,,	104	144	24	9	128	48
55 ,,	72	96	—	10	136	56
				10½	128	
After 10th inj.:						
3 min.	280	256	120			
5 ,,	280	240	64			
30 ,,	232	240	56			
55 ,,	208	200	16			
24 hr after start	13	5.7	0.5	24 hr after start	8	<.05

patient treated with caronamide and in one, in the absence of the drug.

No toxic effects were noted in any of the cases studied. Bleeding and coagulation times and clot retraction remained normal.

It is apparent from these experiments that higher peak levels are obtained and that higher interim levels are maintained by the hourly rapid injection of a concentrated solution of crystalline penicillin than by giving the same amount of the antibiotic during the same period by means of the usual slow continuous method of intravenous administration. In addition, a summation effect is indicated by the levels recorded after the first, fifth and tenth injections in patients No. 5 and 6 who received caronamide. This indicates that the repeated hourly intravenous injection of such large amounts either still further reduced the renal excretion rate or resulted in tissue saturation so that the fall of blood level by movement of the penicillin into the tissues was reduced.

The high levels of penicillin in the peripheral blood, just described, are the result of the hourly and rapid intravenous administration of extremely high dosage in combination with inhibition of renal excretion by caronamide. The intraaortic peak levels are many times higher than those in the peripheral blood when the antibiotic is injected rapidly in concentrated solution. It is believed that even higher sustained levels could be safely achieved by larger single doses.

Studies are now in progress to determine the effect of such high sustained levels in the peripheral blood and high aortic levels of penicillin upon the rapid sterilization of the vegetations in bacterial endocarditis and upon other acute and chronic local and general infections caused by refractory microorganisms. It is also important to determine the effectiveness of this method in shortening the duration of therapy in these diseases and, perhaps, reducing the total amount of penicillin required.

Summary. A method for obtaining high sustained blood levels of penicillin is described. This consists in repeated large intravenous doses of crystalline penicillin, injected rapidly, in patients receiving caronamide. Clinical applications are suggested.

TABLE I.

Effect of Caronamide upon Penicillin Blood Levels in Units per ml in Patients Receiving a Single Large Dose of Crystalline Penicillin (One Million Units).

| | Rapid Intravenous Injection | | | | | | Intramuscular inj. | |
| | Patient 1 | | Patient 2 | | Patient 3 | | Patient 4 | |
Time after inj.	No caronamide	With caronamide	No caronamide	With caronamide	No caronamide	With caronamide	No caronamide	With caronamide
1 min.	112	152	88	152	136	160	—	—
3 ʼʼ	132	152	112	152	152	160	—	—
5 ʼʼ	120	136	112	136	128	144	32	32
10 ʼʼ	104	88	—	112	88	136	32	40
15 ʼʼ	64	72	40	72	56	96	40	—
20 ʼʼ	—	—	—	—	—	—	40	48
30 ʼʼ	40	56	32	48	32	80	32	48
1 hr	16	48	16	40	8	48	24	48
2 ʼʼ	5.7	13	6.6	20	4.4	20	10	20
3 ʼʼ	3	10	2	13	0.8	13	4.4	13

obtained from the descending aortic arch during operation, in a human subject weighing 58 kg were determined. At 1, 3, 5, and 10 minutes following rapid injection into the left antecubital vein of one million units of crystalline penicillin (17,300 units per kg) dissolved in 5 cc of distilled water the levels were found to be 480.0, 346.0, 320.0 and 106.6 units per ml respectively.

The peripheral venous blood levels were then studied in human subjects under the following conditions: one million units of crystalline penicillin dissolved in 5 cc of distilled water were injected rapidly into 3 patients prior to and, again later, following the administration of caronamide. For comparison the same dose was given intramuscularly in a control patient, also preceding and following caronamide administration. The results are given in Table I. In all patients receiving caronamide the dose employed was 4 g orally every 3 hours day and night starting 24 hours before antibiotic therapy and continuing throughout the period of observation.

As may be seen from Table I, a very high blood level still persisted one hour after each injection, especially when the patient was receiving caronamide. The peak level in the venous blood from the opposite arm was reached within the first 3 minutes and was equally high in patients under caronamide therapy and without it. There was a progressive fall during the 3 hours of observation, the most precipitous drop occurring during the first 30 minutes. At the 3-hour period the level in the patients receiving caronamide was 5 to 15 times higher than in the same patients without caronamide.

It was thought that the high level still present at the end of the first hour could be maintained or exceeded if the intravenous injection of the same dose was repeated hourly. Table II shows the levels achieved in 3 patients receiving rapid intravenous injections of one million units of penicillin hourly for 10 hours. Two of these patients received caronamide and one did not. Table II also shows the blood levels found after administration of the same total dose (10 million units in 10 hours) by slow intravenous drip in a

<div align="center">

TABLE I.

Electrophoretic Analysis of Plasma Proteins.

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Case No.	Normals						Experimentals					
	Alb. A	Globulins					Alb. A	Globulins				
		α_1	α_2	β	Φ	γ		α_1	α_2	β	Φ	γ
1	58.5	5.71	7.38	10.71	5.98	11.83	57.3	6.42	7.74	11.84	5.76	11.00
2	56.6	5.80	9.20	11.10	5.50	11.90	57.5	4.10	9.47	13.38	6.31	9.28
3	57.2	2.96	9.98	11.60	8.90	9.70	53.5	3.00	10.70	14.40	8.75	9.70
4	66.8	4.46	8.00	11.24	3.42	6.09	66.9	6.37	7.33	10.85	3.82	4.77
5	57.8	5.41	10.80	11.94	5.40	8.80	57.8	4.53	11.83	14.04	4.76	7.48
6	67.8	4.24	8.47	9.54	3.18	6.71	64.2	6.60	8.80	9.74	4.07	6.61
7	55.9	4.86	10.77	12.15	6.25	10.08	56.0	5.73	7.29	12.50	6.25	11.97
8	64.6	4.98	6.64	11.00	4.67	8.15	61.0	6.67	9.60	11.92	4.36	6.38
9	61.1	5.85	11.99	8.77	6.43	5.85	62.7	4.94	10.64	7.99	5.70	7.98
10	61.1	3.97	10.11	11.55	4.33	9.04	63.7	4.24	8.50	11.89	3.68	7.92
11	61.7	3.40	7.71	9.25	5.24	12.67	61.4	4.78	7.97	9.56	4.78	11.56
12	58.7	3.30	10.19	12.28	5.09	10.48	55.9	4.54	9.41	11.37	7.79	11.04
13	68.3	5.40	7.04	11.22	3.85	4.16	66.6	7.47	7.78	9.98	3.74	4.36
14	58.3	4.39	9.86	11.23	7.12	9.04	56.0	5.06	9.11	12.16	7.60	10.13
15	67.7	5.32	9.88	9.51	2.66	4.94	67.7	4.30	9.41	8.06	2.42	8.05
16	62.3	5.30	13.26	9.47	5.69	6.06	61.2	6.08	11.02	11.79	5.30	4.56
Avg	61.5	4.70	9.45	10.78	5.23	8.46	60.6	5.30	9.13	11.34	5.32	8.29
Std. Dev.	4.17	0.90	1.70	1.08	1.52	2.51	4.31	1.07	1.32	1.90	1.68	2.39

Std. Dev. of Changes*   A α_1 α_2 β Φ γ
                               1.45 0.83 1.26 0.97 0.76 1.09

* Calculated for each component based on difference between normal and experimental values.

was sufficient to fill the apparatus completely. Electrophoresis was carried out in the Longsworth[10] modification of the Tiselius[11] apparatus. The patterns were photographed and projections (2.5X) were traced on bond paper. The area enclosed by the base line and the protein peaks was measured with a planimeter and equated to total protein. Each peak was measured separately to determine the individual proteins. Mobility measurements were made from the boundary anomaly of the pattern. While both ascending and descending boundaries were compared and analyzed, only data from the descending boundaries is included.[10] Changes in total protein content were checked refractometrically, and standardized against a sample of crystalline serum albumin dissolved in the buffer. No set of samples (normal and experimental) showed a greater than 0.5% variation in protein content by this method.

Conditions of the electrophoretic experiments were carefully controlled. The current was held at a value of 25 ± 0.5 milliamperes,

and was applied for 120 ± 1 minutes. The maximum variation of refractive increment was ± 0.0003. The conductivity of each solution was also measured, and the greatest difference observed in any pair of samples was ± 0.0004 ohms⁻¹. In measurements of area by the planimeter, the sum of the areas or the peaks due to protein was 100 ± 0.1%. This agreed with the measurement of the total area within the same limits.

Results. The detailed analysis of each experiment is presented in Table I. Average values agree well with those of other workers,[12,13] and the standard deviations are also in fairly good agreement. Differences between our results and those of others is largely due to the fact that we used the Pedersen[14] method of area division while others have used the method of Tiselius and Kabat.[15] The

[10] Longsworth, L. G., *Chem. Rev.*, 1942, **30**, 323.

[11] Tiselius, A., *Trans. Faraday Soc.*, 1937, **33**, 524.

[12] Dole, V. P., *J. Clin. Invest.*, 1944, **28**, 708.

[13] Bieler, M. M., Ecker, E. E., and Spies, T. D., *J. Lab. Clin. Med.*, 1947, **32**, 132.

[14] Svedberg, T., and Pedersen, K. O., *The Ultracentrifuge*, p. 296, Oxford University Press, London, 1940.

[15] Tiselius, A., and Kabat, E. A., *J. Exp. Med.*, 1939, **69**, 119.

16154

Effect of Acetylsalicylic Acid Ingestion on Electrophoretic Patterns of Human Plasma.[*]

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In their work with the anti-clotting agent dicoumarol, Link et al.[1] presented *in vitro* evidence that salicylic acid was a degradation product of the widely used antipyretic and analgesic acetylsalicylic acid. Following the work of Link, Field[2] discovered that lactating rats, which normally have high levels of fibrinogen and prothrombin, suffered from hypoprothrombinemia after ingestion of dicoumarol; if dicoumarol was replaced by acetylsalicylic acid a much less severe hypoprothrombinemia resulted. Rapoport and Guest[3] found a decreased sedimentation rate of erythrocytes in subjects receiving salicylic acid or its acetyl derivative, but admitted that their results may have been influenced by certain uncontrolled factors. They contended that the chief factor in regulation of sedimentation rates was plasma fibrinogen. This statement was confirmed by Allen et al.;[4] however, these investigators found no hepatic pathology in dogs fed fatal doses of dicoumarol. It was claimed by Homburger[5] that salicylates produce a fall of plasma fibrinogen proportional to total dose rather than to blood levels. His results are subject to question since they are based on a study of only 3 patients, and these had metastatic carcinomas. It has also been claimed by Shapiro, Redish, and Campbell[6] that the ingestion of acetylsalicylic acid increases the clotting time of the blood.

All of the work cited above was based either on the method of Quick[7] or of Cullen and Van Slyke.[8] There are definite theoretical disadvantages in both of these. Quick's method measures clotting time, and depends on the addition of calcium and thromboplastin to a diluted plasma. The dilution is unphysiological and is also subject to salt errors.[9] In Cullen's method a fibrin precipitate is weighed and analyzed for nitrogen; the results are expressed as either prothrombin or fibrinogen. Since the stoichiometry of fibrin formation *in situ* is not known, the method is at best somewhat equivocal.

It appeared to us that electrophoresis might be applied advantageously to this problem, since it is capable of separating the plasma proteins with a minimum of denaturation and loss. While prothrombin is not ordinarily present in sufficient concentration to appear on an electrophoretic pattern, fibrinogen is a well defined component of such patterns.

Materials and Methods. Samples of oxalated blood were obtained from normal healthy male and female subjects before and after a 7-day period during which they ingested 4 g of acetylsalicylic acid per day. The plasma was diluted with 3 volumes of a barbiturate buffer (pH = 8.63, ionic strength—0.1, sodium diethyl barbiturate—0.1N), and dialyzed at 2°-6°C for 3 days with daily change of buffer. Dialysis was performed in Visking casings. The final volume of buffer

1 Link, K. P., Overman, R. S., Sullivan, W. R., Huebner, C. F., and Scheel, L. D., *J. Biol. Chem.*, 1943, **147**, 463.

2 Field, J. B., *Am. J. Physiol.*, 1945, **143**, 238.

3 Rapoport, S., and Guest, G. M., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 43.

4 Allen, E. V., Barker, N. W., and Waugh, J. M., *J. Am. Med. Soc.*, 1942, **120**, 1009.

5 Homburger, F., *Am. J. Med. Sciences*, 1946, **211**, 346.

6 Shapiro, S., Redish, M. H., and Campbell, H. A., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 12.

7 Quick, A. J., *J. Am. Med. Soc.*, 1938, **110**, 1658.

8 Cullen, G. E., and Van Slyke, D. D., *J. Biol. Chem.*, 1920, **41**, 587.

9 Deutsch, H. F., and Gerarde, H. W., *J. Biol. Chem.*, 1946, **160**, 381.

what extent there is renal function in fetal rabbits and to attempt to measure the change in the renal excretion at birth and during the first few days of postnatal life. The rabbit was selected as our experimental animal because of the convenience of its breeding habits, its short gestation period and the fact that, like man, it has only a vestigial allantois.[4]

Methods. Phenolsulfonphthalein was selected as our indicator of renal function because it is rapidly excreted by tubular secretion as well as glomerular filtration.[3] It has been widely used since 1910 as a clinical test of renal function in man and is relatively non-toxic.[2] Finally the method of quantitative estimation of this dye in body fluids is simple and accurate.

A number of does were bred, each by a single copulation, in order that young of known age could be obtained. Some of these does were anesthetized before term and the fetuses exposed while others were allowed to deliver in order that newborn of various ages could be obtained. In this manner a series of fetuses and newborn of known age covering the period from the 26th to the 42nd day after conception was obtained. Deliveries occurred between the 30th and the 33rd days after conception.

A subcutaneous injection of one mg of PSP in a volume of 0.5 ml was adopted as the test dose. The percentage of this dose excreted into the bladder in one hour was determined.

In the fetal series 4 mothers were lightly anesthetized with 1.3 g of urethane per kilogram of body weight augmented with ether during the surgery. The uteri were exposed and a small opening made in each horn. Through these openings the urogenital papillae of 2 fetuses were tied off and the PSP

injections made subcutaneously with special care to avoid leakage. Then the uterine incisions were sutured and the abdomen closed during the collection period. At the end of the hour the fetuses were removed, sacrificed and the bladder contents washed out for analysis. The survival of the fetuses as indicated by pulsation of the umbilical artery was taken to be a proof of the continued normal function of the placenta.

In the newborn series there were 11 litters. Tests of the rate of excretion of PSP were made at daily intervals after birth. Each young rabbit was injected subcutaneously with the standard dose of PSP and suspended over a beaker. At the end of an hour each was sacrificed, the bladder contents added to any urine voided during the hour and analysed for PSP.

Nine experiments were carried out to determine the placental transmission of PSP from maternal to fetal blood and vice versa. In one series the mother was injected intravenously with 6 mg of PSP in one ml and the fetal blood tested after an interval of one hour for the presence of the dye. In the other series 2 fetuses were injected subcutaneously, each with 3 mg of PSP in 0.5 ml volume. The mother was catheterized and urine collected for a period of one hour during which several maternal blood samples were taken. The maternal and fetal blood samples were obtained by heart puncture.

The concentration of PSP in the centrifuged urine was determined with the aid of a photoelectric colorimeter on the properly diluted urine alkalinized with sodium carbonate. The plasmas were first precipitated with one part of saturated trichloracetic acid to 8 parts of blood, the supernatant portion alkalinized and the color estimated. This method measures only the fraction of the phenol red which is not bound to proteins but avoids the errors accompanying hemolysis.

To test the influence of the anesthetic on the renal excretion of the dye, some of the newborn were given 1.3 g of urethane per kg and the rate of their excretion of PSP simultaneously compared with that of an unanesthetized litter mate.

[1] Barclay, A. E., Franklin, K. J., and Pritchard, M. M. L., *The Fetal Circulation*, Charles C. Thomas, Springfield, Ill., 1945.

[2] Rowntree, L. G., and Geraghty, J. T., *J. Pharm. and Exp. Therap.*, 1910, **1**, 579.

[3] Smith, H. W., *The Physiology of the Kidney*, Oxford University Press, New York, 1937.

[4] Windle, W. F., *Physiology of the Fetus*, W. B. Saunders Co., Philadelphia, 1940.

latter of these methods introduces errors discussed by Svensson.[16]

Discussion. Our results indicate that for the 16 cases studied, none of the protein components accessible to electrophoresis shows any marked change. Particularly, only 4 of the 16 cases showed a loss of 0.5% in fibrinogen, while as many cases showed a gain of the same magnitude. Witts[17] has published the statement that fibrinogen levels may be reduced 30% before prothrombin time is significantly prolonged. With respect to the earlier claims cited above, our results indicate that the change in clotting time and sedimentation rates following acetylsalicylate ingestion is not due to marked changes in fibrinogen levels. Actually, the presence of any large asymmetric molecule can change sedimentation rate regardless of protein concentration.[18,19] Whether this is due to an effect on protein surface charge or to chemical interaction is not known. Smith[20] claims that salicylates are bound to some plasma proteins. Ham and Curtis[21] have reported a very careful study of the relation between hepatic function and plasma proteins. These authors concluded that plasma fibrinogen was an especially sensitive index of liver disease or dysfunction. They stated that in damaged livers the plasma fibrinogen was sharply decreased. If these reports are correct then our results indicate that salicylates are not hepatotoxic, and likewise, any effect of salicylates on prothrombin time is not reflected by corresponding changes in fibrinogen levels.

Summary. 1. Blood of normal subjects was examined by electrophoresis before and after ingestion of 4 g of acetylsalicylic acid per day for 7 days. Physical and chemical constants obtained were in good agreement with published values.

2. No marked changes were observed in any protein components accessible to electrophoresis. The conclusion is drawn that the effect of salicylates on blood clotting time and sedimentation rates of erythrocytes cannot be due to a reduction of plasma fibrinogen, and that salicylates are probably not hepatotoxic.

16 Svensson, H., *Ark. Min. Kemi och Geol.*, 1946, 22A, 1.

17 Witts, L. J., *J. Path and Bact.*, 1942, 54, 516.

18 Zozaya, J., Proc. Soc. Exp. Biol. and Med., 1937, 36, 182.

19 Yardumian, K., *Am. J. Clin. Path.*, 1937, 7, 105.

20 Smith, P. K., Gleason, H. L., Stoll, C. G., and Ogorzalek, S., *J. Pharm. and Exp. Therap.*, 1946, 87, 237.

21 Ham, T. H., and Curtis, F. C., *Medicine*, 1938, 17, 413, 447.

16155

Development of Renal Function in Fetal and Neo-Natal Rabbits Using Phenolsulfonphthalein.*

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At birth mammals undergo tremendous physiological adjustments in their respiration, nutrition, circulation and excretion correlated with the achievement of independence of the material circulation. Much attention has been directed toward the first 3 of the above mentioned adjustments but fewer physiological studies have been made on the development of an independent renal excretion. Most of the work reported indicates that there is some formation of urine in the terminal stages of uterine life.[1,2,3] It has been the purpose of our investigations to try to determine to

of glomerular filtration and tubular secretion even in the mesonephros, but his work does not indicate the relative quantity of materials excreted in the urine. That this avenue of excretion is not important so long as the placenta functions normally is indicated by the observation that fetuses can survive to term without kidneys or with obstructed urinary passages.[4,6,8]

In considering the cause of the rather abrupt change in renal function which we have observed at the time of birth, one must consider both physiological and morphological factors. Hamilton, Woodbury and Woods[9] have measured the blood pressures of fetal and newborn rabbits. They find some increase in the systemic arterial pressure at the time of birth and a gradual continuing increase to the adult level. This seems to be true of other mammalian fetuses as well.[1,4,6] †

On the morphological side there are reports that the glomeruli undergo changes at the time of birth which render them more capable of filtration.[6,10] Gruenwald and Popper suggest that the fully developed visceral layer of Bowman's capsule impairs filtration in the embryonic kidney.[10] They believe that a rupture of this epithelial layer occurs at

birth with an expansion of the capillary loops of the glomerulus.

The combined effect of the increase in blood pressure and the increase in filtering surface may cause the sudden increase in renal function observed at birth. The dye then excreted would represent the sum of that filtered at the glomeruli and that secreted by the tubule cells, the dye secreted by the tubule cells being flushed out by the fluid filtered at the glomeruli.

Our failure to find any appreciable placental transmission of PSP in one hour is at variance with the finding of Lell and Liber.[11] These investigators found that 15 to 30% of a dose of PSP injected into rabbit fetuses could be recovered from the urine of the mother in a 6-hour period. This discrepancy with our results may be due to the difference in the time interval if it is not due to differences in the technique of injecting the fetuses.

Summary. 1. Renal function in the rabbit as measured by phenolsulfonphthalein excretion increases rapidly and regularly in the first 10 days after birth but does not exist to an important degree before birth. 2. At the end of 10 days of postnatal life the rate of dye excretion is 100 times greater than the prenatal rate while the renal mass is increased less than 20 fold. 3. In one hour PSP does not traverse the rabbit placenta in either direction in appreciable amounts after the 26th day of gestation. 4. The effect of urethane anesthesia on the excretion of PSP by newborn rabbits was found to be negligible.

7 Gersh, I., *Contr. Emb.*, 1937, **26**, 33.

8 Potter, E. L., *J. Pediat.*, 1946, **29**, 68.

9 Hamilton, W. F., Woodbury, R. A., and Woods, E. B., *Am. J. Physiol.*, 1937, **119**, 206.

† Another possibility is suggested by the work of Truetta *et al.* (*Lancet*, 1946, **251**, 237) who have described a physiological mechanism for diverting the renal blood flow from its normal course in such a way as to render the renal cortex ischemic.

10 Gruenwald, Peter, and Popper, Hans, *J. Urol.*, 1940, **43**, 452.

11 Lell, Wm. A., and Liber, K. E., *Anat. Rec.*, 1928, **38**, 53.
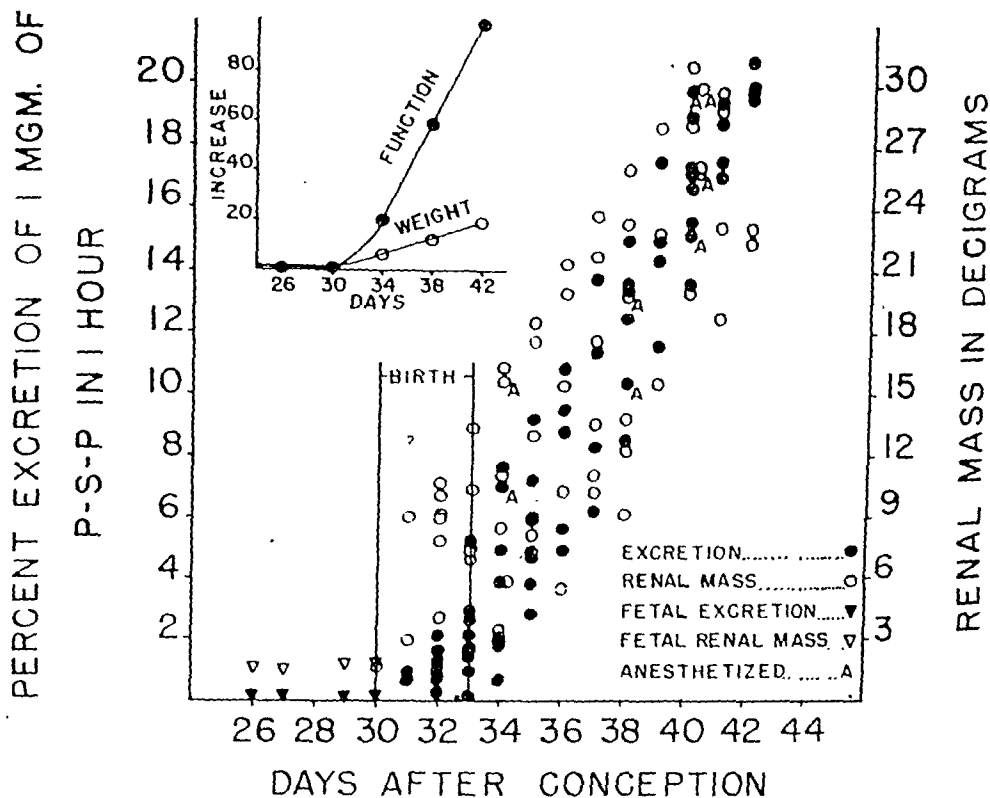
FIG. 1.

The rate of excretion of phenolsulfonphthalein and the combined weights of the 2 kidneys in fetal and newborn rabbits of varying age. The rate of excretion (solid dots) is plotted as the percentage of a 1 mg subcutaneous dose excreted in one hour. The kidney weights (circles) are plotted in decigrams against the ordinate on the right. The absolute rate increase of excretion and renal mass is plotted in the small graph at the upper left, using earliest noted fetal excretion and renal mass values as bases.

The kidneys of fetuses and newborn were removed and weighed after the excretion rate had been determined.

Results. The excretion of PSP by the fetuses in one hour was practically negligible in spite of high plasma concentrations of the dye and showed very little change between the 26th and the 30th day of gestation. (Fig. 1) The weight of the fetal kidneys also showed little change during this period.

Following birth there was a regular increase in the rate of excretion of PSP and in the kidney weight with increasing age. The increase in function was about 5 times greater than the increase in renal mass, *i.e.* the rate of dye excretion at the end of 10 days postnatal life was 100 times greater than the prenatal rate while the renal mass was in-

creased less than 20 fold.

In the experiments comparing litter mates, one of which was anesthetized, there was virtually no effect of the anesthesia on the rate of dye excretion. (Fig. 1)

There was no appreciable transmission of the dye across the placental barrier in either direction during one hour.

Discussion. It is well established that some renal excretion occurs in mammalian fetuses as they approach term.[4,5,6] Gersh has defined qualitatively the beginning of renal excretion in rabbit and other mammalian fetuses by histochemical technique.[7] He found evidence

5 Daly, Harriet, Wells, L. J., and Evans, Gerald, PROC. SOC. EXP. BIOL. AND MED., 1947, 64, 78.

6 Smith, C. A., *The Physiology of the Newborn Infant*, Charles C. Thomas, Springfield, Ill., 1945.
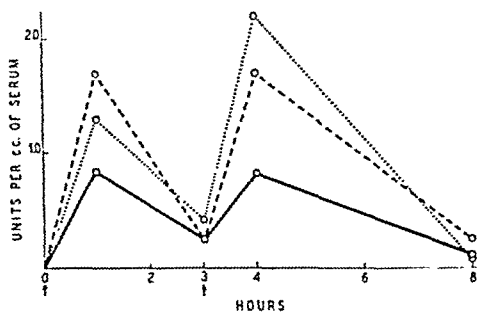
FIG. 1.

Serum concentrations following intramuscular injections of 1000 u/kg in each of 3 dogs. Bacitracin was injected at 0, 3, and 8 hours.

experiment, blood samples taken from each dog were analyzed for their bacitracin content. The data, shown in Fig. 1. indicate that measurable concentrations of the drug following the 3 intramuscular injections persisted in the circulating blood for approximately 13 hours of that day, leaving the blood free of circulating bacitracin for about 11 hours out of the 24.

At the end of the experiment all animals were sacrificed. Macroscopic examination of organs and tissues revealed no significant changes other than local induration at the sites of repeated intramuscular injection.

Microscopic examination of the tissues revealed the following: In the kidney, the cells of the proximal and distal convoluted tubules showed eosinophilia of the cytoplasm. Occasional basophilic globoid bodies were found in the convoluted tubules and a moderate number in the loops of Henle and collecting tubules. An occasional collection of round and plasma cells was found in the stroma. In one dog (No. 156) there was a coagulum in the loops of Henle and the collecting tubules.

The liver appeared to contain considerable glycogen. There was no evidence of cellular damage. The bone marrow was hyperplastic. The spleen showed large follicles and the pulp contained numerous macrophages with brown pigment and megakaryocytes. No significant changes were found. The thyroid appeared hyperplastic. The colloid was vacuolated and in places basophilic. Small nests of epithelial cells were found in dog No. 156. The muscle at the site of injection showed edema and

numerous polymorphonuclear leukocytes which extended between the muscle fibres.

Experiments in Monkeys. After a control period of one to 3 weeks, during which time routine blood counts and urinalyses were performed, 5 monkeys were given bacitracin. Monkeys No. 1 and No. 44, each weighing 3 kg, were given 1500 units of bacitracin, lot No. B-100, per kg of body weight morning and evening for 5 days of each week. On weekends the total dose of 3000 units per kg was given at one time. All injections were given into the posterior muscle group of the right thigh, thus concentrating any local effect there might be. In order to compare the effect of 2 different lots of bacitracin, monkeys No. 2, No. 30, and No. 46 were similarly dosed with bacitracin lot No. B-102. The animals in both groups were dosed for 37 and 39 consecutive days, respectively.

During the period of treatment, blood counts were performed at approximately weekly intervals. The red cell counts showed no significant variation from those of the control period. One monkey, No. 1, showed a definite leukopenia. This animal, at autopsy, exhibited widespread nodular lesions, even though all animals were tuberculin negative at the outset of the experiment. The white counts in the other 4 animals remained in the normal range. During the period of treatment 3 of the 5 monkeys showed a fluctuating, but definite eosinophilia. In contrast with the normal data reported by Downey[4] all animals during both the control and treatment periods showed a lymphocytosis.

Urine samples were collected in metabolism cages and were, therefore, subject to errors of contamination. Nevertheless, it is of interest to note that the urine samples were negative for albumin and sugar during the control period, but after the animals had been on test for 3 weeks, albumin appeared in the urine of 4 of the 5 monkeys. Sugar appeared in the urine of all 5 animals in amounts graded qualitatively from 1 to 3 plus.

Blood sugar (71 and 62 mg %) and non-

[4] Downey, H., *Handbook of Hematology*, P. B. Hoeber, New York, 1938.

16156

Some Pharmacological Characteristics of Bacitracin
III. Chronic Toxicity Studies of Commercial Bacitracin in the Dog and Monkey.*

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In earlier studies of the toxicity of bacitracin,[†] nephrotoxic changes were oberved in the mouse, but not in the rat.[1] In further studies of the absorption, distribution, and excretion of bacitracin in the dog,[2] no apparent clinical signs of renal toxicity were observed. Because of these differences, it appeared desirable to study the toxicity of bacitracin further. The effects of prolonged daily administration of the antibiotic to dogs and monkeys were, therefore, investigated.

Comparison of a number of samples of commercial bacitracin clearly indicated that the toxicity varied independently of the activity[1] and since the toxicity was not altered by destruction of the active principle, it seems reasonable to assume that further purification of bacitracin will lead to a lowered toxicity. Sample No. B-100 was much less toxic than the others and was therefore used for the most part in the tests described below.

Experiments in Dogs. Three mongrel dogs, which had been given bacitracin previously in the course of absorption and excretion studies,[2] each weighing between 7 and 9 kg were maintained on a stock diet of Friskies[‡]

dog meal and water *ad libitum* for a period of 28 days. During this period, control blood counts were performed on each dog at approximately weekly intervals. The data obtained were within normal limits. During this control period, samples of urine were negative for sugar and albumin, and microscopic examination of the sediment was negative. After this period of observation, 1000 units[3] per kg body weight of bacitracin, lot No. B-100, were administered intramuscularly 3 times daily for 5 days of each week. On Saturdays and Sundays 2 doses of 1500 units per kg each were given. The antibiotic, in 2 to 3 cc of water, was injected intramuscularly using a different limb for each injection. The animals received treatment for 24 days. During this period, blood counts were performed at approximately weekly intervals. The red cell count showed no significant variations from the normal. One dog exhibited a moderate, persistent leukocytosis (15,000 per mm[3]) with an increase in the polymorphonuclear leukocytes (average, 87%). The other dogs showed normal total leukocyte counts with an increase in the polymorphonuclear leukocytes (85%). Urinalyses disclosed no significant abnormalities. Blood sugar (80 to 95 mg %) and non-protein nitrogen analyses (30 to 48 mg %) after 10 and 23 days of dosing, disclosed no significant changes except in one dog in which the non-protein nitrogen ranged from 55 to 66 mg %. Without control observations, this cannot be unequivocally attributed to the influence of the antibiotic. There were no appreciable changes in body weight. On the last day of the

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† Bacitracin is the antibiotic discovered by Johnson and associates.[3] The material used in this study was kindly furnished by Dr. John T. Goorley of the Ben Venue Laboratories, Bedford, Ohio.

1 Scudi, J. V., and Antopol, W., PROC. SOC. EXP. BIOL. AND MED., 1945, **64**, 503.

2 Scudi, J. V., Clift, M. E., and Krueger, R., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 9.

‡ Carnation Company, 450 Seventh Avenue, New York, N.Y.

3 Johnson, B. A., Anker, H., and Meleney, F. L., *Science*, 1945, **102**, 376.

shaved abdominal skin of each of 5 rabbits with no visible reaction within the ensuing 4 days.

A solution of 1200 units of bacitracin per cc of normal saline at pH 7.0 was instilled into the conjunctival sac of rabbits and the lids were held closed for 2 to 5 minutes. Even though the residual solution was not washed out, only faint evidences of irritation were noted in each of the 5 rabbits used. The faint reddening of the conjunctiva disappeared within 4 hours in all animals.

It is to be noted that the foregoing results were obtained with relatively impure commercial bacitracin concentrates as currently produced. Somewhat different results may be obtained with pure materials.

Summary. 1. Following prolonged daily administration of crude bacitracin concentrates in dogs and monkeys, no significant changes in blood morphology were observed. 2. Injection of bacitracin solution (6000 units per cc) into the shaved abdominal skin, and instillation of bacitracin (1200 units per cc) into the conjunctival sac of the rabbit caused little irritation. 3. Repeated intramuscular injection of 1000 units of bacitracin per kg 3 times each day for 23 days into the dog produced local induration. 1500 units per kg twice a day for 45 days in the same area in monkeys produced both induration and small areas of necrosis. 4. In the dog, urine samples remained negative for sugar and albumin while in the monkey, sugar and albumin appeared in the urine as the animals continued on test. 5. Large doses of bacitracin, approximating the LD_{50}, produced damage to the renal tubules, with tubular necrosis in the mouse. In the rat and the dog, the lesions were, insignificant, while in the monkey, necrotic cells were found in two instances only, and then in comparatively insignificant numbers as contrasted with the mouse.

16157

Comparison of Intestinal Lengths and Peyer's Patches in Wild and Domestic Norway and Wild Alexandrine Rats.[*]

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The two most common species of rats in the world, the Norways and the Alexandrines or roof rats, have very different resistance to poisoning with alpha naphthyl thiourea (ANTU) and show very different symptoms. Alexandrines have an LD_{50} of 250 mg/kg which is more than 30 times as high as the LD_{50} of the Norways, 6.9 mg/kg.[1] Furthermore, in the latter species ANTU produces a marked pulmonary edema and pleural effusion, while in the former species it causes no detectable change in the lungs or in any other organs.[2] At present these species differences in toxicity and physiological effects remain unexplained. The observation[2] that herbivorous animals such as rabbits, guinea pigs, meadow mice, prairie dogs, ground squirrels, and monkeys have a high resistance to ANTU poisoning and show no lung effects, while carnivorous or omnivorous animals such as dogs and pigs do show a marked pulmonary edema and pleural effusion and a relatively low resistance, may throw some light on this problem,[†] The Alexandrine rat superficially resembles the Norway rat; often inhabits the same houses and buildings; and in general

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[1] Dieke, S. H., and Richter, C. P., Proc. Soc. Exp. Biol. AND MED., 1946, **62**, 22.

[2] Richter, C. P., J. A. M. A., 1945, **129**, 927.

protein nitrogen (31 and 32 mg %) analyses in 2 monkeys just prior to the termination of the experiment were within the normal range, (sugar 60-80 mg % and non-protein nitrogen 30-40 mg %). Approximately 2 to 5 hours after the last dose of the drug just before the animals were sacrificed, samples of blood and spinal fluid were withdrawn and analyzed for their bacitracin content. The blood concentrations were 6.6, 0.75, 0.18, 8.1, and 6.6 units per cc and the spinal fluid concentrations were 0.34, .009, .08, and .34, and .34 units per cc, respectively, indicating that the antibiotic does not readily pass the blood brain barrier in the normal monkey.

Gross examination of the organs revealed in monkey No. 1, firm grey nodules 1 to 5 mm in diameter in the lungs, liver, spleen, kidney, and heart. The kidneys of the other monkeys were moderately enlarged and edematous. The cut surface lipped over the capsular edge. The capsule stripped with ease, revealing a smooth surface. The kidneys exhibited a diffuse deep yellow color with an orange tint. The muscle at the site of injection was edematous and, in places, there were small areas of necrosis. No other gross abnormalities were noted.

Microscopic examination of the kidney in the monkeys disclosed prominent epithelial elements in the glomeruli; and, in some cases, these contained an abundant clear cytoplasm. The cytoplasm of the convoluted tubules was eosinophilic (hematoxylin-eosin stain). There was coagulum in the loops of Henle and the collecting tubules; at times, globoid bodies were present. In the loops of Henle some of the cells were vacuolated. In the tubules of monkeys No. 1 and No. 2 necrotic cells could be found only very infrequently. No necrotic cells were found in the kidneys of any of the other monkeys. There was an occasional collection of round cells in the medulla. No excess fat could be demonstrated by Sudan stains in frozen section. Best's carmine stain revealed no glycogen.

The liver contained considerable glycogen. No cellular damage was seen. The spleen showed no significant changes. The bone marrow was hyperplastic. In the lung of monkey No. 44 there were foci of a sub-acute pneumonia. The testes showed no spermatogenesis. Since the age of the animals is unknown, this finding cannot be evaluated. The thyroid in monkey No. 1 showed slight glandular hyperplasia. The intestine in monkey No. 30 was ulcerated and showed a granulomatous reaction in the wall, and parasites resembling oxyuris were found in the lumen. In the adrenals, the reticularis was dense. The muscle at the site of injection contained collections of round cells and polymorphonuclear leukocytes which extended for a considerable distance between the muscle fibres. Small areas of necrosis were present. In monkeys No. 1 and No. 2, sarcosporidia were found.

In monkey No. 1 the nodules in the lung, liver, spleen, kidney, and heart were granulomatous and contained numerous epithelioid cells. No acid-fast bacilli or other organisms could be demonstrated in the tissues with Ziehl-Neelsen stain. The etiological factor for these lesions was not ascertained.

In contrast with the striking kidney lesions in the mouse,[1] the kidney in the rat[1] and the dog did not vary appreciably from the normal. The renal lesions in the monkey were insignificant in comparison with those of the mouse.

Additional Experiments. Since bacitracin is harvested from bacterial cultures, it was of interest to determine whether or not the crude materials at hand were anaphylactogenic. Accordingly, attempts were made to sensitize each of 6 guinea pigs with 5 mg (150 units) of bacitracin administered subcutaneously as a single dose. One month later a shocking dose of 10 mg (300 units) was administered intracardially with no evidences of anaphylaxis, but additional experiments would be required to demonstrate that none of the phenomena of anaphylaxis can be caused by bacitracin.

Bacitracin solution (lot B-100 at a concentration of 6000 units per cc of distilled water) was injected intradermally in doses of .05 and .10 cc into different areas of the
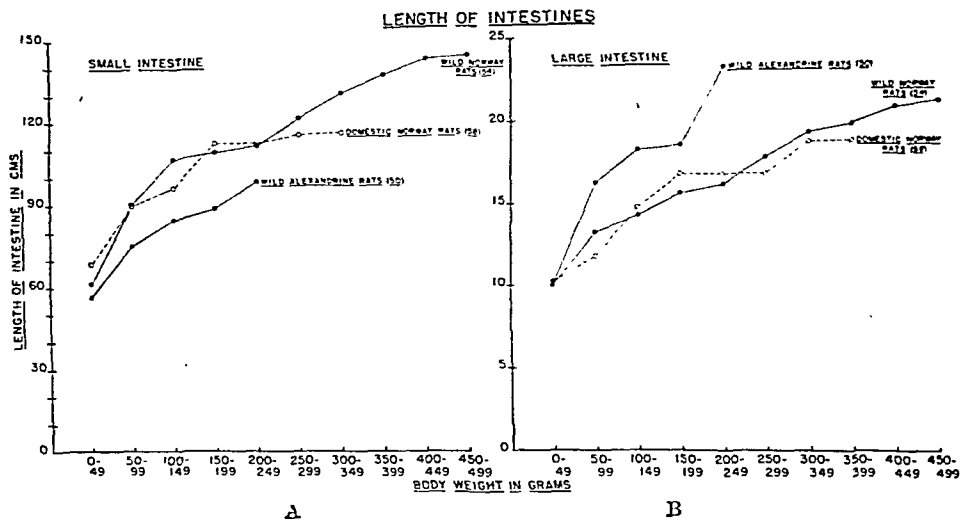
FIG. 1.
Graph showing the relation between the length of the gastro-intestinal tract and body weight.
1A. Small intestines.   1B. Large intestines.

FIG. 2.
Photograph of typical Peyer's patch.   Intestine filled with hematoxylin.

domestic Norway 349 g; and the heaviest Alexandrine 248 g.

Fig. 1a shows that the Norway rats, both wild and domestic strains, had definitely longer small intestines than did the wild Alexandrines. In the 200-249 g range the small intestines of the Norways had an average length of 113 cm while those of the wild Alexandrines averaged only 98 cm. The small intestines of the domestic Norway were of essentially the same length as those of its wild congener in all weight ranges investigated up to 300 g.

Figure 1b shows that the reverse relationship holds for the length of the large intestines. The wild Alexandrines had definitely longer large intestines than did either the wild or domestic Norways. For the 200-249 g weight group the length of the large intestine averaged 23.0 cm for the wild Alexandrines, and only 16.0 and 16.5 cm respectively for the wild and domestic Norways. Here again the

also lives in close association with man and man's food. It may, however, belong to the herbivorous group of animals listed above, or at least when compared to the Norway it may have a more herbivorous diet. Many workers who have observed the Alexandrine rat in the field believe that it does have a predominately herbivorous diet.

To throw some light on these questions we have now undertaken several comparative studies on Alexandrine and Norway rats. Some of these studies are anatomical, concerned with physical characteristics, while others are behavioristic, concerned with dietary selections. The present report deals with a comparison of the gross anatomical characteristics of the intestinal tracts of these two species of rats; the lengths of the small and large intestines and the number of Peyer's patches on the small intestines. Intestinal lengths were selected for examination since it is well known that in general herbivores have longer large intestines than do carnivores or omnivores, and often shorter small intestines.[3,4] The laboratory or domestic Norway differs anatomically and physiologically in a number of ways from its wild Norway counterpart, so it was also included in the present study. Our observations were limited to the wild form of Alexandrine rats because these rats have not been domesticated.

Methods. The wild Norway rats were trapped in Baltimore and surrounding farms by the City Rodent Control Department and the Rodent Ecology Division of the School of Hygiene of the Johns Hopkins University. Almost all of the wild Alexandrine rats came

from ships in the Baltimore harbor where they had been killed by fumigation.[‡] Some of the domestic Norways came from our laboratory colony, descendants of a colony of Wistar rats established 20 years ago. Others came from the Carworth Farms, and still others from Dr. E. V. McCollum's colony. The wild and domestic Norways were killed with ether. Within one hour afterwards the intestinal tract was removed, freed from all connections, washed, and evacuated. It was suspended over a strip of cross-section paper and then both were laid flat on a table. The location of the pyloric and cecal ends of the small intestine and the cecal and rectal ends of the large intestines were marked on the paper and measured. The location and size of the Peyer's patches were also indicated by dots on the chart paper. The size of the dots was made to conform roughly to the size of the Peyer's patches. For the Alexandrine rat the technique was much the same except that the rats were killed with cyanide gas and were not always autopsied within the first few hours after death; in a few instances they were not autopsied until 18 hours post-mortem. When not autopsied immediately they were kept in a refrigerator. To give a clear definition to the Peyer's patches, particularly in very young rats, the intestines were in some instances filled with a 1% solution of hematoxylin which stained all of the walls of the intestines except the intestinal patches; in other instances the rats were given olive oil by stomach tube several hours before they were killed.

Observations were made on 54 wild Norways; 58 domestic Norways; and 50 wild Alexandrines. Each group contained approximately the same number of males and females.

Results. Intestinal Lengths. Fig. 1 summarizes the results of the observations made on intestinal lengths of the two strains of Norway rats. The ordinates give intestinal length in centimeters; the abscissae body weight in grams. The rats were fairly evenly distributed over the total body weight range. The heaviest wild Norway that we could get for this study weighed 499 g; the heaviest

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† Cats, which definitely belong to the carnivorous group of animals, show all of the lung effects that are found in rats, pigs, and dogs, but they do not have as low an LD_{50}, at least when they receive the ANTU by stomach tube. Their ready ability to vomit may explain the higher LD_{50}. We have not determined the parenteral LD_{50}.

3 Buddenbrock, W. von, *Grundriss der vergleichenden physiologie*, Berlin, 1928, p. 650.

4 Dukes, H. H., *The Physiology of Domestic Animals*, 4th edition, Comstock Publishing Co., 1937.

‡ Dr. M. F. Haralson, in charge of the Quarantine Station of the City of Baltimore, kindly supplied us with these rats.

ent was comparable to that found in adult rats.

Discussion. The results show that compared to the omnivorous or carnivorous Norway rats, the Alexandrine rat has a definitely shorter small intestine and a longer large intestine. Thus, according to these findings, the Alexandrine rat either belongs to the herbivorous group of animals, or at least when compared to the Norway it must have a much more herbivorous diet. It should thus be able to utilize larger amounts of cellulose material.

These findings in themselves do not prove that the Alexandrine rat is an herbivore. They will have to be substantiated by the results of dietary selection studies. They do indicate, however, that the high resistance of the Alexandrine rat to ANTU poisoning and its lack of any lung effects may depend on some factors which the herbivorous animals have in common. The observation that the Alexandrine rats have only about half as many Peyer's patches as do the Norways has an interest quite apart from any light that it might throw on the question whether or not the Alexandrine rats belong to the herbivore. The Peyer's patches on the intestines have been counted in a number of different animals, horses, cows, sheep, pigs, dogs, cats and rabbits. They range in number from 2-4 for cats to 180-320 for horses.[5] Kelsall[6] recently counted the number of patches in different strains of mice and found that the means varied from 6.3 in the C57 black strain to 10.7 in the C3H strain, while intermediate groups including strains C, Swiss, and dba, had 8-9 Peyer's patches. Clearly, in number of patches the mice show a much closer relationship to the Alexandrine rats (which had an average of 8.7 patches) than they do to the Norways with an average of 16-18 patches.

Mice as well as the Alexandrine rats appear to prefer an herbivorous diet. They live largely on grain, flour, and cereal. This suggests that a correlation may exist between the dietary habits and the number of Peyer's patches, or indirectly the total amount of lymphoid tissue in the body. An association of a lower amount of lymphoid tissue with an herbivorous diet however would not agree with the well-known fact that in general, herbivorous animals have more lymphoid tissue than do carnivorous animals.[7] This discrepancy may result from the assumption that the number of Peyer's patches bears a direct relation to the total amount of lymphoid tissue in the body. Although in general appearance, behavior and habits, mice appear to be more closely related to Alexandrine rats than they do to Norways, they have a lower resistance to ANTU poisoning than do Alexandrines, and also show pulmonary edema and pleural effusion.

Kelsall[6] reported a direct correlation "between the amount of intestinal lymphoid tissue, as measured by the number of Peyer's patches, and the incidence of spontaneous mammary tumors." She reported that in the strain of mice which had only an average of 6.3 patches the incidence of tumors was very low, while in the breeding females of those with an average of 10.7 patches it was 95%. On this basis the Norway rats with their much higher number of Peyer's patches should exhibit a higher incidence of tumors than do the Alexandrines. Our results show that domestic Norway rats have more Peyer's patches than do the wild Norways. We have no explanation for this finding. Previous experiments have revealed other differences between the wild and domestic rats.[8-12] In this connection one of the most interesting differences is that the domestic rats have much smaller adrenal glands.[12] This recession of

[5] Patzelt, V., *Handb. der Mikro. Anat. des Mensch.*, Julius Springer, Berlin, 1936, **5**, 218.

[6] Kelsall, M. A., Proc. Soc. Exp. Biol. and Med., 1946, **61**, 423.

[7] Ehrlich, W. E., *Ann. New York Acad. Sc.*, 1946, **46**, 823.

[8] Dieke, S. H., and Richter, C. P., *J. Pharm. and Exp. Therap.*, 1945, **83**, 195.

[9] Fish, H. S., and Richter, C. P., Proc. Soc. Exp. Biol. and Med., 1946, **63**, 352.

[10] Griffiths, W. J., Jr., *Science*, 1944, **99**, 62.

[11] Griffiths, W. J., Jr., *Am. J. Physiol.*, 1947, **149**, 135.

[12] Rogers, P. V., and Richter, C. P., in press.
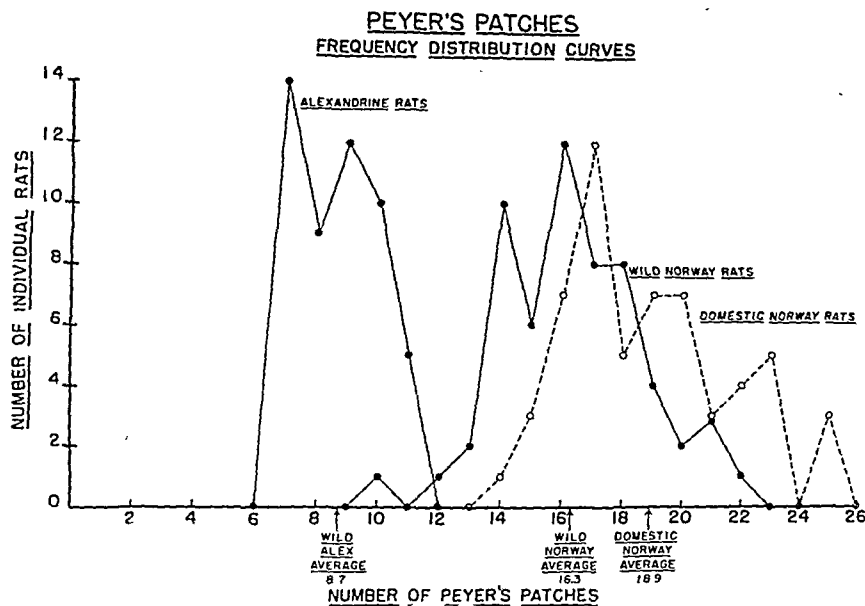
FIG. 3.
Chart showing frequency distribution curves of Peyer's patches.
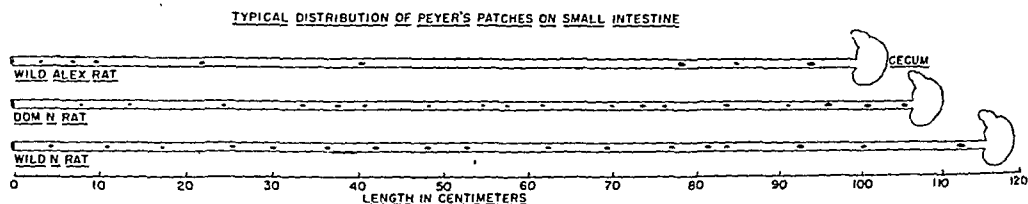
FIG. 4.
Drawing of 3 intestinal tracts from wild Alexandrines, domestic Norways, and wild Norways respectively, showing distribution of Peyer's patches.

wild and domestic Norways had essentially the same intestinal lengths in all weight groups.

Peyer's Patches. Fig. 2 shows a photograph of a Peyer's patch which is fairly typical for the two Norway strains and for the wild Alexandrines. The intestine of this rat was filled with the hematoxylin solution. This patch contained 13 follicles.

Fig. 3 gives the frequency distribution curves for the number of Peyer's patches found in the 3 types of rats. It shows that the Norway rats had many more patches than did the Alexandrine rats. For the Alexandrines the average number and its standard error were 8.7 ± 0.19, for the wild Norway 16.3 ± 0.31, and for the domestic Norway 18.9 ± 0.37. The curves for the wild Alex-

andrines and Norways showed almost no overlap at any point. Fig. 4 shows a typical distribution of Peyer's patches on the small intestines of a wild Alexandrine, domestic Norway and wild Norway respectively. The sizes of the patches are drawn roughly to scale. In the wild Alexandrine most of the patches were usually found near the two ends of the intestine, while in the 2 Norway types the patches were distributed at fairly regular intervals throughout the entire length of the intestines. For all the 3 types of rats the patches tended to become much larger in the lower third of the intestines. We were not able to establish any relationship between number of Peyer's patches and age or sex. At an age of approximately 15 days when the patches first became visible the number pres-

Effect of Varying the Interval Between Hypophysectomy and Gonadotrophin Injection on Ovulation in the Rat.

Hrs from hypophysectomy to injection	No. of rats hypophysectomized	No. of rats ovulated
0	6	6
2	6	5
4	9	8
6	8	7
8	12	11
10	14	6
12	8	1
19	6	0
24	5	0

duct, newly formed corpora lutea, and pre-ovulatory follicles.

Results and Discussion. The results as summarized in Table I show that in a high percentage of the animals tested the mature follicles retain their ability to ovulate in response to an intravenous injection of chorionic gonadotrophin provided the injection is made within 8 hours after the hypophysectomy. If the interval between hypophysectomy and injection is extended to 10 or more hours, the percentage of animals which will respond declines rapidly with no animals responding after 12 hours.

The data show that the pituitary is necessary for maintaining the mature follicles found in early proestrus so that they will ovulate in response to a gonadotrophic stimulus. They also indicate that after hypophysectomy the amount of the pituitary hormones responsible for the maintenance drops rapidly to a level in the blood below that required to keep the follicles in a condition where they can be ovulated.

It was found that the follicles in the ovaries of the rats injected 8 to 12 hours following hypophysectomy which failed to ovulate often showed a marked degree of luteinization, but when the interval was extended to 19 hours or more luteinization did not occur.

Summary. After an interval of about 10 hours following hypophysectomy in early proestrus, the mature follicles in the ovary of the rat become refractory to a single intravenous injection of human chorionic gonadotrophin but the ability of the follicle to luteinize is retained for a longer time.

16159 P

Statistical Evaluation of Growth Curves.

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In an article with the above title which appeared in this journal,[1] Weil compares the growth curves of groups of experimental animals subject to different treatments. Five treatments are compared against each other and a control, 30 rats are used for each treatment and the weight of each rat is determined at weekly intervals. Weil proposes a method of analysis in which he constructs a frequency distribution for each treatment taking all the weights of the rats over the period of the experiment and then applies the chi-square test to examine differences between those frequency distributions for the various treatments.

This is an oversimplified method of analysis and not a valid one. The fallacy lies in the tacit assumption that all the observations within each of the frequency distributions are independent. Although the 30 rats in each group are independent, the repeat weighings on each rat are certainly not so. In practically all biological work, the main source of variability lies between animals:

[1] Weil, C. S., PROC. SOC. EXP. BIOL. AND MED., 1947, 64, 468.

the adrenals might have resulted in an increase in lymphoid tissue, since it is well known that after removal of the adrenals the thymus and other lymphoid tissue hypertrophies.

Summary. 1. Alexandrines or roof rats (*Rattus rattus*) have shorter small intestines and longer large intestines than do Norway rats (*Rattus norvegicus*). 2. The intestines of Norway rats have about twice as many Peyer's patches on the average as do those of Alexandrine rats, averaging 16-19 as compared to 9.3. The intestines of domestic Norway rats have more Peyer's patches than do those of wild Norways. 4. Peyer's patches first become visible when the rats reach an age of 15 days. The number does not appear to change with age.

16158 P

Effect of Interval Between Hypophysectomy and Injection of Gonadotrophin on Ovulation in the Rat.*

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Hertz and Meyer[1] have shown that if young adult female rats are hypophysectomized during the first 2 hours of proestrus ovulation is prevented during the subsequent 46-hour period. It was concluded that a hypophyseal hormone (LH) is required for ovulation in the rat and that the quantity required is secreted subsequent to early proestrus.

The work reported here was undertaken to determine how long after hypophysectomy mature follicles retain their ability to ovulate in response to a single intravenous injection of human chorionic gonadotrophin, and is preliminary to a series of experiments designed to study the hormonal requirements for normal development of follicles and ova just prior to ovulation.

Method and Materials. The 72 rats employed in this experiment were 3- to 5-month-old virgin females of the Sprague-Dawley strain.

All hypophysectomies were carried out during the first 3 hours of proestrus using virtual-

ly the same technique described by Hertz and Meyer.[1]

The intravenous injections of the purified human chorionic gonadotrophin, prepared essentially by the method of Gurin, *et al.*,[2] were given in one of the tail veins with the exception of a few injections which were made into the heart. Fifty and 100 γ doses of the dry powder in an alkaline saline solution at a concentration of 100 γ/cc were given but since the results for the 2 doses were comparable, they have been combined in Table I. The timing of the injections in relation to hypophysectomy is also shown in this table.

Autopsies were carried out 30 to 50 hours after the injections allowing ample time for all the ova which had ovulated to reach the oviduct. The sella was examined with magnifying spectacles to determine if the hypophysectomy had been complete. The ovaries and oviducts were removed and fixed in FAA or Zenker's solution.

Serial sections of one ovary and oviduct from each rat were made and stained with hematoxylin and eosin. The sections were then examined for the presence of ova in the ovi-

1 Hertz, Roy, and Meyer, R. K., PROC. SOC. EXP. BIOL. AND MED., 1946, 63, 71.

2 Gurin, S., Bachman, C., and Wilson, D. W., J. Biol. Chem., 1939, 128, 525.

16160 P

An Experimental Study of the Cerebral Coproporphyrin in Rabbits.[*]

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Klüver's discovery of the presence of small amounts of coproporphyrin in the central nervous system of warm-blooded animals[1,2] raised the question whether there might be a relationship between this porphyrin and the increased urinary coproporphyrin in conditions such as lead and arsenic poisoning, which are characterized both by injury of the nervous system and by marked coproporphyrinuria. It is well known that in heavy metal and chemical poisoning the excess urinary coproporphyrin is the type III isomer.[3] Preliminary studies by Klüver,[4] and in this laboratory, with the differential precipitation, or "fluorescence quenching" technique[5,6] indicate that the coproporphyrin of the nervous system is likewise the type III isomer. Hence it seemed desirable to determine the coproporphyrin concentration in the brains of rabbits suffering from acute lead poisoning.

Hitherto, quantitative data on the coproporphyrin of the central nervous system have not been reported, nor has a quantitative method of determination been described. The method devised for use in the present study combines certain features of procedures previously described for urinary coproporphyrin[6] and erythrocyte protoporphyrin.[7] The procedure was briefly as follows: The entire rabbit brain was ground in a mortar, washed repeatedly with physiological saline solution to remove most of the blood, and then mixed with 10 ml of glacial acetic acid and 100 ml of acetone. The mixture was allowed to stand overnight with repeated shaking. It was filtered through cheesecloth and the residue was ground with additional amounts of glacial acetic acid and acetone, and finally with ethyl acetate.[†] The residue was pressed as dry as possible. The combined filtrate was mixed with an equal volume of distilled water and extracted 4 times with ethyl acetate. The latter was extracted 4 times with 15 ml portions of 10%HCl. The acid extract was made negative to Congo red paper by addition of saturated aqueous sodium acetate solution, and the solution was extracted 4 times with ethyl acetate after a few ml of glacial acetic acid had been added. The combined ethyl acetate extracts were washed with water and

TABLE I.
Cerebral Coproporphyrin in Normal Rabbits.

No.	Wt of brain in g	γ of coproporphyrin (Total content)	γ/g brain
1	8.1	.3	.037
2	9.1	.225	.025
3	8.5	.57	.067
4	8.3	.30	.036
5	8.0	.27	.034
6	7.6	.345	.045
7	9.0	.225	.025
8	8.7	.225	.026
9	8.0	.30	.038
10	7.7	.18	.023
		Avg	.0356

1 Klüver, H., *J. Psychol.*, 1944, **17**, 209.

2 Klüver, H., *Science*, 1944, **99**, 482.

3 Watson, C. J., and Larson, E. A., *Physiol. Rev.*, 1947, **27**, 478.

4 Klüver, H., personal communication.

5 Schwartz, S., Hawkinson, V. E., and Watson, C. J., *Science*, 1946, **103**, 338.

6 Schwartz, S., Hawkinson, V. E., Cohen, S., and Watson, C. J., *J. Biol. Chem.*, 1947, **168**, 133.

7 Grinstein, M., and Watson, C. J., *J. Biol. Chem.*, 1943, **147**, 675.

† More recently it has been found advantageous to grind and extract the brain with glacial acetic acid and ethyl acetate (1:10), on a sintered glass filter; several such extractions obviate the necessity of preliminary extraction with acetone and the period of standing in contact with it. (C. J. W.)

repeat determinations per animal will result in more precise measurement for the individual animals, but will not reduce the effect of the basic variability between the animals. Applying the chi-square test in the way proposed by Weil may grossly overestimate the significance of the differences between the treatments.

There are valid methods of comparing growth curves of the type considered by Weil. These are indicated in broad outline in this note and a more detailed account with numerical examples will be published elsewhere.

In his paper, Weil considers the t-test applied to the weights after a given time, e.g. weights after 12 weeks. Provided the apportionment of the animals between the groups has been carried out in a strictly random manner, this method of test is valid. It may not be the best since it does not make use of the earlier weighings. Some improvement would probably result if the final weights are corrected for the variations in the initial weight of the rat (or any other characteristic which may be correlated with the final weight). The method to use here is the covariance method discussed by Fisher.[2]

A more complete method would be to fit regression lines, such as by the method of least squares, to the growth curve of each rat using, if required, a simple transformation to the time scale and weight scale in order to produce a simple curve. We may apply the method of the previous paragraph to estimates of the weight of rat obtained from the fitted lines at any given time. We could go further if desired and assess the significance of the differences between the constants of the fitted curves. For example, in the case

of the slope of the curve, we adjust this slope (if required) for the estimated initial weight of each rat and calculate the significance of the effect of treatments on the adjusted slopes by Fisher's method already referred to. If the growth curves have been transformed into straight lines, this would represent a complete analysis.

Sufficiently approximate results may often be obtained from curves fitted by eye. We could compare any property of the growth curves between groups, e.g., rate of growth at any given time, increase in weight between any given times, etc.

When the growth curves cannot be conveniently transformed into straight lines, these methods may have the disadvantage of not measuring the overall differences between the growth curves. A satisfactory method would be to read off from the fitted curves (by eye or by calculation) the estimated weights at the initial time and at two other times chosen to give a fairly adequate description of the growth curves. We then apply the method of "discriminant function analysis"[2] to these pairs of weights. This gives the maximum discrimination between the treatments and at the same time, furnishes a satisfactory test of the significance of the differences between the treatments. When it is desirable to correct for variations in the initial weight of the rats, we can apply this correction and the discriminant function analysis simultaneously.

With, if necessary, the use of a simple transformation for either or both of rat weight and time, it is nearly always possible to give an adequate description of the growth curves from 2 or 3 points. In rare cases a fourth point might be necessary. The discriminant function analysis can be extended quite readily to accommodate another point.

2 Fisher, R. A., Statistical Methods for Research Workers, Ninth Edition, Oliver & Boyd, Ltd., Edinburgh and London, 1944.

16161 P

Coronary Sinus Catheterization Technique for Studying Coronary Blood Flow and Myocardial Metabolism *in vivo.*

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Recent reviews stress the need for a method of studying the coronary circulation in intact animals, as well as in man.[1,2] Harrison and coworkers cannulated the coronary sinus of intact morphinized dogs,[3] with a modified Morawitz brass balloon cannula.[4] A less traumatic technique of intravenous catheterization of the coronary sinus of intact dogs is presented in this report, using the soft Forssman catheter,[5] as modified by Cournand.[6] Application of this method in studying myocardial metabolism *in vivo* will be reported elsewhere,[7,8] particularly the measurement of coronary blood flow by the nitrous oxide method, developed by Kety and Schmidt for measuring cerebral blood flow.[9]

The catheter,[6] (size 7-9, reasonably stiff but without stylet), is inserted through the dog's external jugular vein, under light nembutal anesthesia. Fluoroscopically, in the right anterior oblique position,* a triangular area of lung with the following boundaries is visible: (1) the anteromedial border of the inferior vena cava, (2) the posteroinferior cardiac border, and (3) the diaphragm. The coronary sinus ostium lies just anteromedial to the superior corner of this triangle which marks the junction of inferior cava and right auricle, posteroinferior to the tricuspid valve. The catheter is first passed into the inferior cava, then withdrawn just inside the auricle. As the tip is shifted anteromedially, with repeated gentle thrusts toward the ostium, the catheter will eventually enter the coronary sinus, (Fig. 1). The tip often passes further in the same direction, superiorly and to the left along the auriculo-ventricular groove, past a delicate valve into the great cardiac vein. Sometimes the catheter enters the middle cardiac vein, or more rarely the first posterior vein of the left ventricle, and passes along the posteroinferior septal surface toward the apex.

Evidence of successful coronary sinus catheterization includes: (1) the typical fluoroscopic position of the catheter,† (2) withdrawal of very dark venous blood which shows an extremely low oxygen content, markedly lower than in mixed venous blood, (Table I), and (3), in some cases, autopsy with the catheter still inserted in the sinus.

The coronary sinus of 30 dogs, weighing 28-75 lbs., has been successfully catheterized 55 times, with as many as 7 procedures on the same dog at monthly intervals. Three attempts in small dogs were failures. Postoperative recovery was prompt, except in those intentionally sacrificed. No post-

1 Gregg, D. E., *Physiol. Rev.,* 1946, **26**, 28.

2 Ratnoff, O. D., *Medicine,* 1946, **28**, 285.

3 Harrison, T. R., Friedman, B., and Resnick, H., Jr., *Arch. Int. Med.,* 1936, **57**, 927.

4 Morawitz, P., and Zahn, A., *Zentralbl. f. Physiol.,* 1912, **26**, 465.

5 Forssman, W., *Klin. Wchnschr.,* 1929, **8**, 2085.

6 Cournand, A., *Fed. Proc.,* 1945, 207.

7 Eckenhoff, J. E., Hafkenschiel, J. H., Harmel, M. H., Goodale, W. T., Lubin, M., Bing, R. J., and Kety, S. S., in press.

8 Goodale, W. T., Lubin, M., and Banfield, W. G., in press.

9 Kety, S. S., and Schmidt, C. F., *Am. J. Phys.,* 1945, **143**, 53.

* Terminology, as in *man.*

† Diodrast (3,5-diiodo-4-pyridone-N-acetic acid and diethanolamine) was occasionally injected forcibly to outline the coronary venous system fluoroscopically, but sometimes caused local myocardial necrosis and hemorrhage. Forcible injection of any fluid against the coronary venous stream may be hazardous.

TABLE II.
Urinary and Cerebral Coproporphyrin in Lead-Poisoned Rabbits.

No.	UCP* in γ/day at time of killing	Wt of brain in g	γ of coproporphyrin (total content)	γ/g brain
1	12.6	7.5	.3	.04
2	16.0	9.1	.48	.052
3	20.0	8.0	.195	.024
4	3.7	8.5	.30	.035
5	11.5	9.3	.195	.021
6	19.8	9.2	.345	.037
7	43 0	8.3	.15	.018
8	58.0	8.6	.195	.023
9	49.0	8.0	.345	.043
10	44.0	9.1	.345	.038
11	15.0	7.7	.30	.040
12	26.0	8.0	.405	.05
				Avg .035

* UCP = total urinary coproporphyrin.

extracted 4 times with 2-3 cc portions of 1% HCl. This was washed with $CHCl_3$, separated and filtered. The determination of total coproporphyrin was then made on the 1% HCl extract in the Klett fluorophotometer, as previously described.[6] The concentration and individual content was determined for the brains of 10 normal rabbits, data for which are given in Table I.

Twelve rabbits were poisoned with lead acetate, the first 6 receiving 50 mg per kilo, the second six 100 mg per kilo, in a single intraperitoneal injection. The animals were killed 7-10 days later. The analytical data are given in Table II. The urinary coproporphyrin was determined by the method of Schwartz and associates[6] which gives an upper limit of 5 γ per day for normal rabbits.

No increase in the brain coproporphyrin occurred following the acute lead poisoning.

Thus it is evident that the present study does not provide evidence of a relationship between the coproporphyrin content of the brain and that of the urine which was much increased. The possibility cannot be excluded that an accelerated formation and release of coproporphyrin by the brain could have maintained the actual concentration at a constant level. Other studies are in progress in which various substances affecting the nervous system are being used, both in acute and chronic experiment.

Summary and Conclusions. 1. A method is described for the quantitative determination of coproporphyrin in the brain. 2. The concentration and total content of the coproporphyrin of normal rabbit brains was compared with that of brains from rabbits, in which acute lead poisoning was induced. No variation was observed.

TABLE I.
Oxygen Content of Blood Samples.[15]

	Oxygen content vol. %				
	No. of observations	Mean value	S.E.*	S.D.†	C.V.‡	Extreme range
Coronary sinus	44	3.8	.16	1.04	27.7	2.4- 8.3
Pulmonary artery or rt. ventricle	30	12.5	.72	3.89	31.5	9.5-15.3
Femoral artery	48	16.9	.25	1.75	10.3	14.5-22.6

* Standard error of the mean.
† Standard deviation.
‡ Coefficient of variation.

izing the pulmonary artery or right ventricle.‡ The auricular lesions were more frequent and more severe than those found after passing the catheter only into the coronary sinus.

In 3 cases, lesions obviously peculiar to coronary sinus catheterization were found: 2 of coronary venous thrombosis and one of gross hemorrhage into the myocardium drained by a catheterized vein. These were perhaps related to prolonged insertion of a large catheter beyond the sinus into the great cardiac vein, or to forceful reinjection of fluid through the catheter. Such lesions have not been found in 11 control experiments in which precautions were taken, including the gentle insertion of a No. 7-F catheter only 1-2 cm into the coronary sinus for only 60 minutes. With these precautions, coronary sinus catheterization appears to be actually less hazardous than catheterization of the right ventricle or pulmonary artery of the dog by our technique.

In man, several thousand well-controlled intracardiac catheterizations have been performed without cardiac damage apparent even in numerous autopsied cases.[6,10-14] Species

peculiarities and differing techniques may well explain the occurrence of lesions in dogs, where none have so far been found in man. The present technique, however, is being applied to current studies of the coronary circulation in man only with the precautions which prevented significant myocardial and coronary venous damage in dogs.

Summary. Coronary sinus catheterization technique has been developed in intact dogs in order to study coronary blood flow and myocardial metabolism, and to evaluate the safety and practicality of a similar procedure in man. The pathological findings, among 30 autopsies following the procedure, have been discussed.

‡The occurrence of endocardial lesions in dogs after catheterizing the pulmonary artery by this technique, has been recently confirmed, although with less frequency and severity than in the present series (Hellems, H. K., Haynes, F. W., Fanger, H., and Dexter, L., personal communication, 1947).

10 Bing, R. J., Vandam, L. D., Gregoire, F., Handelsman, J. C., and Goodale, W. T., PROC. SOC. EXP. BIOL. AND MED., 1947, 66, 239.

11 Dexter, L., Haynes, F. W., Burwell, C. S., Eppinger, E. C., Seibel, R. E., and Evans, J. M., J. Clin. Invest., 1947, 26, 554.

12 Bing, R. J., personal communication.

13 Dexter, L., personal communication.

14 Cournand, A., personal communication.

15 Roughton, F. J. W., and Scholander, P. F., J. Biol. Chem., 1943, 148, 541.
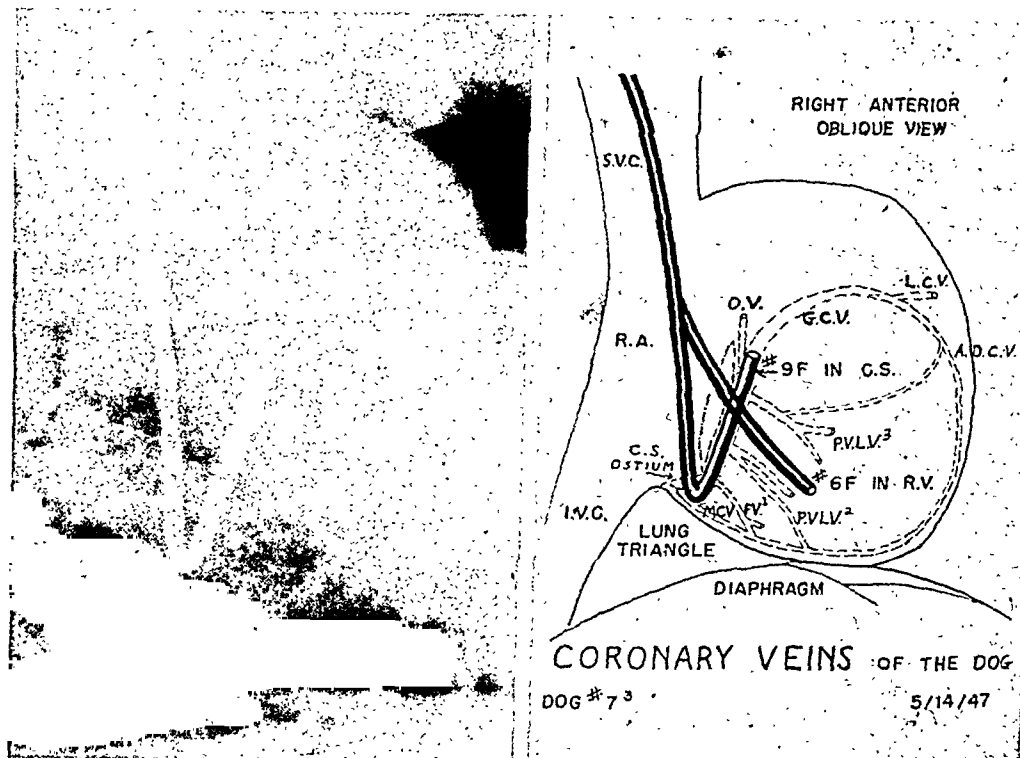
FIG. 1.

Large No. 9 catheter is inserted 3-4 cm into coronary sinus (C.S.), with small No. 6 catheter passing through tricuspid valve (— · — ·), into right ventricle (R.V.). The coronary sinus venous system, as found at autopsy, is sketched in as follows: middle cardiac vein, MCV; posterior veins of the left ventricle, PV_1, $PVLV_2$, and $PVLV_3$; oblique vein of Marshall, OV; great cardiac vein, GCV; left circumflex vein, LCV; anterior descending coronary vein, ADCV. The extensive veno-venous anastomoses between the major veins draining the left ventricle are illustrated. (The venous drainage of the right ventricle is largely independent of the coronary sinus system, through the Thebesian, anterior, and other small cardiac veins.)

Metabolic Observations: 19 Aug., 1947; Oxygen content of blood from coronary sinus, 3.7 vols. %; pulmonary artery, 14.8 vols. %; femoral artery, 18.2 vols. %, 94% saturated. Coronary flow, 71 cc/min/100 g of left ventricle. Myocardial oxygen consumption, 10.6 cc/min/100 g. Systemic blood flow, 168 cc/kg body wt/min. Weight of dog, 31 kg. Heart weight at autopsy, 240 g. Mean arterial blood pressure, 115 mm Hg. Work of heart, $7/6 \cdot QR = 503$ kg/hr. Cardiac efficiency, 16%.

Pathology: This dog was catheterized 7 times in 5 months, with a total of over 16 hours of actual insertion of the catheter in the coronary sinus, with a second catheter in the pulmonary artery or right ventricle. Autopsy 7 weeks after the last procedure showed a normal heart except for slight subendocardial fibrosis in the right auricle and medial tricuspid valve leaflet.

operative local or systemic infection, or other clinical complications attributable to catheterization, were encountered.

Thirty autopsies following coronary sinus catheterization often showed small mural thrombi and subendocardial hemorrhages in the coronary sinus and right auricle. Gross lesions were rarely found when the dog was sacrificed immediately after catheterization, but often when sacrificed at least 24 hours later. Minimal subendocardial fibrosis in the right auricle was the only finding in 4 of 5 dogs sacrificed 3 to 6 weeks after catheterization.

Mural thrombi and subendocardial hemorrhages, however, were also found in the right auricle, on the right ventricle and on the tricuspid and pulmonary valves after catheter-

TABLE I.
Effect of Injections of Desoxycorticosterone Acetate, Thiouracil, and Combination of Both Drugs on Teeth and Eyes of Baby Rats.

	Control 15 rats		DOCA 15 rats		Thiouracil 11 rats		Thiouracil + DOCA 21 rats	
	Mean	σ	Mean	σ	Mean	σ	Mean	σ
Age at eruption of incisors (days)	9.5	0.63	8.4	0.29	11.3	0.61	9.2	0.84
Age at opening of eyelids (days)	15.1	0.43	14.1	0.61	16.9	0.45	15.5	0.73

ployed had no effect on the body weight of rats even though the injections were continued for 23 days. Thiouracil also had little or no effect on the growth curve for the first 10 to 15 days. However, there then occurred a plateauing of body weight so that in the next week, the thiouracil treated rats showed marked stunting of growth and evidence of immaturity in activity and physical appearance. This has been described by Hughes[4] as cretinism. DOCA, despite its antagonistic effect on the thiouracil depression of tooth, mouth and eyelid development, had no effect whatsoever on antagonizing this stunting effect of thiouracil. Their sickly state was not affected by DOCA and occasionally they seemed more depressed than the rats receiving thiouracil alone. One group of rats which received both drugs for a period of 2 weeks was observed further and the weight curve was compared to that of the littermate controls. At the end of 3 weeks without injections the average weight of the rats was about 15% below that of their controls. In another litter, 2 of the 4 rats in a similar group became weak, stopped feeding

and died, despite the cessation of treatment.

The thyroid glands of the thiouracil treated rats were grossly much larger than those of the controls. Histologically they showed enlargement and increased height of the follicle cells. The glands of the animals receiving the combination of DOCA and thiouracil were indistinguishable from those treated with thiouracil alone, whereas those injected with DOCA alone resembled the normal controls.

Comment. There is little evidence from these experiments to indicate that the precocious development produced by DOCA is mediated through the thyroid gland. Although thiouracil depresses the normal thyroid gland function, there is no certainty that it does so completely in the first few injections. If DOCA could produce its usual changes in a surgically thyroidectomized rat, a definite conclusion could be arrived at.

It is of interest, that despite the fact that thiouracil produces marked changes in the development and growth of the rats, DOCA was able to antagonize its effects on tooth eruption and eyelid opening. Whether the same phenomena can be produced by large doses of other steroid hormones and whether other effects of the thiouracil-thyroid deficiency can be corrected by these hormones remains to be studied.

FIG. 1.
Growth curves of baby rats receiving DOCA, thiouracil, or a combination of both drugs. There are 3 rats in each group and all are littermates.

Summary. Thiouracil injections into baby rats resulted in retardation of tooth eruption and opening of the eyelids. Desoxycorticosterone, when injected into thiouracil treated rats, was able to antagonize these developmental effects so that the time of tooth eruption and eyelid opening returned to normal. It had no antagonizing effect, however, on the stunting of body growth or on the histological picture of the thyroid gland produced by thiouracil.

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Effect of Desoxycorticosterone on the Development of Rats Treated with Thiouracil.

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It has been shown that either adrenal cortical extracts or desoxycorticosterone acetate (DOCA) hastened the development of newborn rats.[1] Under the effects of these substances, the rats' teeth erupted earlier and their eyes opened sooner than littermate controls. The mechanism whereby this precocious development takes place is under investigation. It has been reported that thyroxine produced a qualitatively similar hastening of tooth development[2] and experiments were undertaken to determine whether the adrenal cortical action was mediated through the thyroid gland.

Thyroidectomy in the newborn is an extremely hazardous and frequently fatal operation.[3] Depression of the normal thyroid activity can be accomplished in baby rats by means of thiouracil.[4,5] The effects of DOCA on thiouracil treated rats are reported.

Method. Sixty-two one-day-old rats from 6 litters of the Sprague-Dawley strain were divided into 4 groups. Wherever there were sufficient rats the individual litters were divided so that there were at least 3 rats for each of the following experimental conditions. A total of 15 rats received daily (including Sunday) subcutaneous injections of 0.25 mg of DOCA in 0.05 ml of peanut oil. Eleven rats received similar injections of one to 4 mg of thiouracil, suspended in 0.1 ml of peanut oil. Twenty-one rats received daily injections of both drugs, and 15 rats

remained as the untreated control group. Injections were usually given for about 2 weeks. Each litter of rats remained with its respective mother throughout the experiment. Daily body weights were recorded for each rat and observations of tooth development, eyelid opening, and general physical condition were made each morning and night. At the completion of the experiments, the animals were sacrificed and their thyroids were sectioned and stained with hematoxylin and eosin.

Results. All the rats tolerated the injections and handling fairly well in the newborn period. Hair growth at the site of the DOCA injections was sparse in both groups of rats receiving the drug.

Table I reveals the effects of the drugs on the eruption of the incisor teeth and opening of the eyelids. Although figures given are means of results from 6 litters, the results in individual litters were similar. DOCA invariably hastened the eruption of the teeth, stimulated the differentiation and separation of the lips from the gingiva, and hastened the opening of the eyelids. Thiouracil, in doses of one to 4 mg per day, on the other hand markedly depressed all these developmental changes. In those rats receiving injections of both DOCA and thiouracil, a mutually antagonistic effect was observed. The tooth eruption and eyelid opening in these rats was within normal limits. This was confirmed statistically in that the means of the DOCA and thiouracil groups were significantly different from that of the controls, but the mean of the group receiving the combination of both drugs was not significantly different from that of the controls.

The effects on the body weight and growth curve of one representative litter of 12 rats are shown in Fig. 1. DOCA in the dose em-

1 Mulinos, M. G., and Parmer, L. G., *Science*, 1942, 95, 484.

2 Karnofsky, D., and Cronkite, E. P., Proc. Soc. Exp. Biol. and Med., 1939, 40, 568.

3 Salmon, T. N., *Endocrin.*, 1938, 23, 446.

4 Hughes, A. M., *Endocrin.*, 1944, 34, 69.

5 Goldsmith, E. D., Gordon, A. S., and Charipper, H. A., *Am. J. Obst. Gyn.*, 1945, 49, 197.

TABLE I.

Assays of Urine Following the Administration of Pteroic Acid.

Amt administered* mg	Route	Assay result				24-hr urine vol, ml	Apparent excretion of pteroic acid‡		Apparent conversion of pteroic acid to pteroylglutamic acid§	
		S. faecalis R		L. casei			% administered dose	Avg %	% admin. dose	Avg %
		Basal mγ/ml	24 hr sample mγ/ml	Basal mγ/ml	24 hr sample mγ/ml					
2	Oral	2	19	11	19	970	2.7	1.4	Insignificant	
2	"	2	2	8	6	2330	0			
5	"	2	18	5	8	1680	1.7	2.1	"	
5	"	2	46	8	12	890	2.4			
5	"	3	32	9	5	1260	2.3			
10	"	2	4	8	8	1770	0.2	0.3	"	
10	"	4	14	10	13	620	0.4			
2	Intravenous	2	150	6	20	2000	46	33	1.1	1.4
2	"	2	208	5	38	1200	39		1.6	
2	"	2	85	11	36	1170	15		1.4	

* The compound was prepared as described in the text. It was found to be 90% pure by the chemical reduction test‖ and by measurement of the extinction coefficient at 365 mμ. When the preparation was assayed in comparison with pteroylglutamic acid values of 32% and .004% of the activity of pteroylglutamic acid were obtained, respectively, with S. faecalis R and L. casei.

† Calculated in equivalence of pteroylglutamic acid.

‡ Calculated from the assay with S. faecalis R, using a factor of 32% (see above) to convert the pteroylglutamic acid equivalence into terms of pteroic acid.

§ Calculated from the assay with L. casei, since this organism responds to pteroylglutamic acid and not to pteroic acid. The calculation was adjusted for the purity of the pteroic acid and the molar weight ratios of pteroic acid and pteroylglutamic acid.

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Urinary Excretion Studies Following the Administration of Pteroic Acid.

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A number of reports have dealt with the apparent urinary excretion of pteroylglutamic acid as measured with *Lactobacillus casei* or *Streptococcus faecalis* R. In normal subjects on "average diets," the excretion is quite low, usually in the neighborhood of 4 to 5 μg daily. The urinary excretion rises following the administration of a few milligrams of the pure substance. In one investigation, assays with *S. faecalis* R indicated that from 44 to 57% of an oral dose of 5 mg was excreted in six hours.[1]

The name pteroic acid[2] has been given to the portion of the pteroylglutamic acid molecule exclusive of glutamic acid, more specifically 4(((2-amino-4-hydroxy-6-pteridyl)methyl)amino)benzoic acid. It may be synthesized by condensing p-aminobenzoic acid with α-β-dibromopropionaldehyde and 2,4,5-triamino-6-hydroxypyrimidine.[2] Pteroic acid can replace pteroylglutamic acid in promoting growth of certain microorganisms, notably *S. faecalis* R, but it is without appreciable growth-promoting action on *L. casei*.[2] The presence of pteroic acid in the urine would therefore produce a response in the "folic acid" assay when carried out with *S. faecalis* R but would be without effect if the assay were carried out with *L. casei*. No studies have been reported describing the urinary excretion of pteroic acid following its administration and such an investigation was the subject of the present study.

Experimental. Pteroic acid was synthesized as described above* and was obtained in a state of approximately 90% purity as estimated by chemical assay[3] and extinction co-efficient measurements. The compound was administered as a solution of the monosodium salt to normal adult male subjects. The urine was collected under toluene and the samples were assayed for "folic acid" with *S. faecalis* R[4] and for pteroylglutamic acid with *L. casei*.[5] The results are summarized in Table I.

Discussion. Pteroic acid has been found to stimulate the growth of certain lactic acid bacteria under conditions in which a response also occurs to pteroylglutamic acid. In one class of organisms, typified by *L. casei*, a response is produced by pteroylglutamic acid but not by pteroic acid or p-aminobenzoic acid. A second class, typified by *S. faecalis* R, responds to pteroylglutamic acid and pteroic acid, but not to p-aminobenzoic acid. A clue to the function of pteroic acid in this class of organisms may be afforded by the observation[6] that certain enterococci in "resting cell suspensions" convert the "S.L.R. factor," which is pteroic acid with an added formyl group,[7] to pteroylglutamic acid. A third type of organism, exemplified by *L. arabinosus* 17-5[8] responds to either pteroylglutamic acid, pteroic acid, or p-aminobenzoic acid. Studies

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* Kindly supplied by Dr. C. W. Waller.

3 Hutchings, B. L., Stokstad, E. L. R., Boothe, J. H., Mowat, J. H., Waller, C. W., Angier, R. B., Semb, J., and SubbaRow, Y., J. Biol. Chem., 1947, **168**, 705.

4 Landy, M., and Dicken, D. M., J. Lab. Clin. Med., 1942, **27**, 1086.

5 Teply, L. J., and Elvehjem, C. A., J. Biol. Chem., 1945, **157**, 303.

6 Stokes, J. L., and Larsen, A., J. Bact., 1945, **50**, 219.

7 Wolf, D. E., Anderson, R. G., Kaczka, E. A., Harris, S. A., Arth, G. E., Southwick, P. L., Mozingo, R., and Folkers, K., J. Am. Chem. Soc., 1947, **69**, 2753.

8 Lampen, J. O., and Jones, M. J., J. Biol. Chem., 1947, **170**, 133.

1 Jukes, T. H., Franklin, A. L., Stokstad, E. L. R., and Boehne, J. W., III, J. Lab. Clin. Med., 1947, **32**, 1350.

2 Angier, R. B., Boothe, J. H., Hutchings, B. L., Mowat, J. H., Semb, J., Stokstad, E. L. R., SubbaRow, Y., Waller, C. W., Cosulich, D. B., Fahrenbach, M. J., Hultquist, M. E., Kuh, E., Northey, E. H., Seeger, D. R., Sickels, J. P., and Smith, J. M., Jr., Science, 1946, **103**, 667.

16164

Local Resistance to a Lethal Dose of Formalin.[*]

GABRIEL GASIC.[†] (Introduced by E. C. MacDowell.)

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According to Selye[1,2,3] an animal exhibiting an "alarm reaction" in response to a given chemical is able to resist a lethal dose of a chemical of different nature because of a non-specific mechanism of general defense initiated by this "alarm reaction" and called "reaction of adaptation." In repeating certain of Selye's experiments, preliminary to studies on induced immunity to transplantable leukemia, evidence was obtained that questions the general distribution of the cross resistance between the two chemicals employed.

The present communication is concerned with a study of the location of resistance to formalin and adrenalin induced by formol treatment when the sites of pretreatment and lethal doses were varied.

The experimental material consisted of male and female mice of strain C58,[4,5] whose ages varied between 5 and 7 weeks and whose weights varied between 13 and 22 g. These animals received the standard food and care of this laboratory.[6] Solutions of formalin of 4% and 10% (prepared from the commercial solution) and adrenalin in aqueous solution

of 1:1000 were used as "alarming" stimuli in subcutaneous injections. The minimum lethal doses of these substances were found to vary according to the site of injection (Table I).

Treatment previous to the lethal dose was administered within a period of 48 hours, using 4 injections. The doses used when all 4 injections were given at the same site were the following: 0.10, 0.20, 0.30, and 0.40 cc of 4% formalin; 0.05, 0.10, 0.12, and 0.15 cc of 10% formalin; or 0.06, 0.08, 0.10, and 0.10 cc of adrenalin; in different experiments the site was the skin of the abdomen, the dorsolumbar region, or the ventral surface of the left hind leg. When the injections were made at different sites in the same mice, they received either 4 doses of 0.15 or 0.20 cc of 4% formalin, or 4 injections of 0.06 cc of adrenalin, the sites being: the extremities, and the abdominal, anterior thoracic, dorsolumbar, and dorsocervical regions. In a few experiments each dose of the treatment with progressive amounts of the chemicals was divided into 4 subdoses, and

TABLE I.
Minimum Lethal Dose of Formalin and Adrenalin Given Subcutaneously in C58 Mice According to the Sites.
Male weight: 18 to 22 g; female weight: 13 to 20 g.

Chemical and concentration		Dorsolumbar	Abdominal	Anterior thoracic	Medial surface hind left leg	Dorsocervical
Formalin	10%	0.20	0.20	0.20	1.15	0.10
Formalin	4%	0.50	0.50	—	—	—
Adrenalin	1%	0.15	0.12	—	—	—

1 Selye, Hans, *Arch. Internat. de Pharmacodyn. et de Therap.*, 1937, 55, 431.

2 Selye, Hans, *Am. J. Physiol.*, 1938, 122, 347.

3 Karady, S., Browne, J. S. L., and Selye, H., *Quart. J. Exp. Physiol.*, 1938, 28, 23.

4 Gruneberg, Hans, *The Genetics of the Mouse*, London, Cambridge University Press, 1943, p. 326.

5 Richter, M. N., and MacDowell, E. C., PROC. SOC. EXP. BIOL. AND MED., 1929, 26, 362.

6 Laanes, T., *Hand. der biol. Arbeitsmethoden*, Ed. E. Abderhalden, Berlin, Urban und Schwarzenberg, 1936, 9, 7, 593.

of the relative potencies of these 3 compounds for *L. arabinosus*[8] showed that the first 2 are less active on a molar basis than is p-aminobenzoic acid.

In experiments with chicks, pteroic acid[2] and p-aminobenzoic acid[9,10] have been found ineffective as a replacement for pteroylglutamic acid in the diet. Pteroic acid was administered,[11] 5 mg daily for 10 days without effect, to a patient with pernicious anemia in relapse. The patient subsequently responded to treatment with 5 mg of pteroylglutamic acid daily for 6 days.

A study of the urinary excretion of pteroylglutamic acid indicated that about half of the dose, as estimated by assay with S. *faecalis* R, appeared in the urine during 6 hours following the oral administration of 5 mg of the substance. Similar results were obtained when intravenous administration was used.[1] In contrast, pteroic acid when fed by mouth did not appear to be excreted in significant amounts in the urine, but when it was given intravenously a considerable proportion of the administered dose appeared in the urine. This may indicate that the rate of uptake from the gastrointestinal tract is low, or, less probably, that the compound is destroyed in the tract before being absorbed. The results are interesting in view of the low solubility of the monosodium salt of pteroic acid which is in contrast to the moderate solubility of sodium pteroylglutamate. At the pH of the small intestine, the ion formed by the dissociation of only the carboxyl group would be the predominant form of pteroic acid.

A possibility to be considered in the metabolism of pteroic acid is its conversion to pteroylglutamic acid. The respective activities of the 2 compounds for S. *faecalis* R and *L. casei* enable a differential assay to be conducted, especially if pteroic acid is present in amounts considerably in excess of pteroylglutamic acid. In this investigation, the *L. casei* assay value of the urine was very low following the intravenous administration of pteroic acid in contrast to the S. *faecalis* R assay value. This indicated that the urine contained predominantly pteroic acid rather than pteroylglutamic acid. However, a distinct rise in the *L. casei* value was observed, which indicated probable conversion of a small proportion of the pteroic acid to pteroylglutamic acid (Table I).

Summary. 1. Pteroic acid was administered to normal adult human males and the urine was assayed for "folic acid" with S. *faecalis* R and for pteroylglutamic acid with *L. casei*. On the basis of the relative response of these organisms to pteroic acid and pteroylglutamic acid, the apparent concentration of these compounds in the urine was determined.

2. The oral administration of from 2 to 10 mg of pteroic acid resulted in only a very small amount of urinary pteroic acid.

3. Following the intravenous administration of 2 mg of pteroic acid the microbiological assays of the urine indicated that from 15 to 46% of the compound was excreted as pteroic acid and that only about 1% appeared to be converted to pteroylglutamic acid.

4. The excretion studies indicated that only a small proportion of an administered dose of pteroic acid was converted to pteroylglutamic acid and that pteroic acid is poorly absorbed from the gastrointestinal tract.

9 Lillie, R. J., and Briggs, G. M., *Poultry Sci.*, 1947, **26**, 289.

10 Unpublished investigations in this laboratory.

11 Spies, T. D., and Stone, R. E., *Southern Med. J.*, 1947, **40**, 46.

TABLE III.
Anatomical Findings Soon After Treatment with 10% Formalin.
Group I—4 injections of 0.05, 0.10, 0.12, and 0.15 cc.
Group II—4 injections of 0.10, 0.15, 0.20 and 0.25 cc.

Experimental groups	No. of mice	Organ wt (mg)					Local lesion site inj.	General hyperemia
		Liver	Spleen	Thymus	Adrenal	Pancreas		
I	5	726 (600-800)	37 (30-38)	14 (12-18)	5.0* (4.4-5.5)	85 (68-99)	+++	++
II	5	814 (700-950)	46 (38-68)	21 (10-35)	4.5* (4.0-5.2)	95 (60-155)	+++	++
Normal (controls)	5	802 (720-880)	81 (64-127)	47 (41-60)	4.1 (3.8-4.5)	141 (90-179)	—	—

* Most of the animals showed adrenal changes; cortex pink and slightly transparent; pale medulla, serous membrane edema doubtful in Groups I and II after treatment. One spontaneous death in Group I and 3 in Group II.

of untreated controls injected with the same dose (2 out of 35, line 13). The animals treated with adrenalin did not show cross-resistance to formalin, whether the lethal dose was injected at the same or at a different site (lines 10 and 11).

Autopsy was made on various animals in order to investigate the anatomical symptoms of the alarm reaction and the reaction of the skin at the site of treatment (Table III). At the termination of the treatment, involution of the thymus was evident. Changes in the suprarenal and pancreas were less conspicuous, with a few exceptions. General hyperemia was present, and digestive hemorrhages were frequently observed in animals treated with adrenalin.

The skin showed changes only when formalin was used, and edema and congestion were apparent during the treatment; several days after the last injection, formation of a necrotic scar became evident.

Histological examination of animals treated with formalin, kindly made by Dr. Richard Miller, revealed the changes of the thymus mentioned under the description of the alarm reaction, increase of mitosis of the cortical cells of the suprarenal, and a necrotic and inflammatory type of skin alteration.

It appears that the induction of resistance to a lethal dose depends upon the nature of the chemical used and upon the site. The animals pretreated with adrenalin evinced resistance to the lethal dose of adrenalin of general character, at least in the one small experiment, but showed no cross-resistance to formalin, either local or general. On the contrary, the animals pretreated with formalin showed only a local resistance, whether they were subjected to a lethal dose of formalin or one of adrenalin. Although no general resistance was observed, the formalin-treatment produced the principal anatomical signs of Selye's "alarm reaction."

These differences observed in mice treated with formalin and adrenalin appear to have their explanation in the differing capacities of these substances to induce local lesions. While the first caused intense manifestations at the site of the injection, culminating in

TABLE II.

Number of Mice Surviving Lethal Dose of Formalin or Adrenalin When Injected in the Site of Pretreatment or in a Different Site.

	Pretreatments			Lethal dose		No. of mice	No. surviving after					Total surv.
	Chemical	Dosage	Sites	Chemical	Sites		1st injec.	2nd injec.	3rd injec.	4th injec.	L.D.	
1	Form.	incr.	same	—	—	34	34	34	33	33	—	33/34
2	"	"	diff.	—	—	28	28	18	11	3	—	3/28
3	"	"	same	Form.	same	88	87	85	84	74	65	65/74
4	"	"	"	"	diff.	96*	89	92	77	61	7	7/61
5	"	equal	diff.	"	same	31	28	28	26	26†	13	13/22
6	"	"	"	"	diff.	54	37	35	35	35	9	9/35
7	Adren.	incr.	same	Adren.	diff.	16	16	15	13	11‡	5	5/5
8	Form.	"	"	"	same	55	45	45	41	39§	36	36/38
9	"	"	"	"	diff.	26	26	26	26	26	3	3/26
10	Adren.	equal	diff.	Form.	same	16	16	12	8	6	0	0/6
11	"	"	"	"	diff.	10	9	7	4	3	0	0/3
12	Untreated controls					128					11	11/128
13	"			Adren.		35					2	2/35

* One group of 21 animals received the treatment in the same site but each dose was distributed in 4 different sites.
†, ‡, § Only 22, 5, and 38 animals, respectively, were injected with the lethal dose.

administered at 4 different sites. The animals were usually subjected to the lethal test dose (the size of the dose varying according to the site) 12 hours after the last of the pretreatments, although in a few cases the lethal dose was administered 2, 3 or 6 days later. When death occurred, it was, in most experiments, at approximately the same time as the death of the controls without pretreatment.

Resistance to the preliminary treatment and lethal dose of formalin depends upon the site of administration (Table II). When progressive doses of this substance were injected into one site, 33 out of 34 animals resisted the 4th pretreatment (line 1), while there were only 3 survivals out of 28 cases when the 4 treatments were given in different sites (line 2). Resistance to the lethal dose also depends on the same factor. When it was administered at the site of pretreatments, 65 out of 74 mice survived (line 3); when the lethal dose was injected at a different site, only 7 out of 61 survived (line 4), a result similar to the controls without pretreatment (survival, 11 out of 128, line 12).

The pretreatment was less effective when sublethal doses of equal amount were used. Animals treated in this manner showed resistance to lethal dose injected at the same site in 13 cases out of 22 (line 5), and the animals which died did so after a longer interval than the controls which had been pretreated at different sites (survival, 9 out of 35, line 6).

Many of the animals treated with adrenalin died during the course of treatment, whether this substance was administered at the same site or at different ones. Some of them, subjected to a lethal dose after 4 preliminary injections, resisted adrenalin even when it was applied at a site different from that of the pre-treatment (survival, 5 out of 5, line 7).

In experiments with cross-resistance, 36 out of 38 mice pretreated with formalin resisted a lethal dose of adrenalin when all injections were at the same site (line 8); when the lethal dose was injected at another site, only 3 out of 26 survived (line 9), with the result that the survival rate approached that

TABLE I.

The Relationship Between the Amount of dl-Methionine Alpha-amino Nitrogen Filtered and the Amounts Excreted and Reabsorbed in a Normal Dog.

Dog No. III

Exp. No.	Period	Urine flow, cc/min.	Glomerular filtration rate, cc/min.	Alpha-amino nitrogen					
				Plasma conc. mg%	Urine conc. mg%	Filtered mg/min./sq.m.	Excreted mg/min./sq.m.	Reabsorbed mg/min./sq.m.	Ratio Reabsorbed/Filtered
1	Control	0.37		3.9	3.6		0.03		
1% methionine, 0.5% inulin (Wt. 7.7 kg; S.A.,* 0.437 sq.m.)	1	4.57	46.5	9.4	2.1	10.1	0.22	9.88	.98
	2	4.77	46.2	11.4	7.0	12.1	0.77	11.3	.93
	3	6.23	52.2	13.4	11.6	16.0	1.65	14.4	.90
	4	6.37	48.3	15.0	18.1	16.6	2.64	14.0	.84
2	Control	—		—	—		—		
1.3% methionine, 0.5% inulin (Wt. 7.7 kg; S.A., 0.437 sq.m.)	1	2.47	49.8	11.0	12.4	12.5	0.70	11.8	.94
	2	4.63	51.6	12.9	16.9	15.3	1.79	13.4	.88
	3	5.67	44.8	15.1	22.8	15.5	2.96	12.5	.81
	4	5.60	53.1	16.8	35.3	20.4	4.52	15.9	.78
3	Control	0.17		3.5	6.8		0.03		
1.5% methionine, 0.5% inulin (Wt. 8.2 kg; S.A., 0.455 sq.m.)	1	2.63	61.9	10.4	7.6	14.1	0.44	13.7	.97
	2	7.26	65.7	15.5	23.6	22.4	3.77	18.6	.83
	3	7.47	58.9	17.2	30.2	22.3	4.96	17.3	.78
	4	6.67	52.4	18.7	21.0	21.5	3.08	18.4	.86
4	Control	—		—	—		—		
2% methionine, 0.5% inulin (Wt. 9.55 kg; S.A., 0.504 sq.m.)	1	2.57	58.5	11.4	20.7	13.2	1.06	12.1	.92
	2	4.63	58.2	14.7	27.7	17.0	2.54	14.5	.85
	3	7.80	57.5	19.6	34.1	22.4	5.28	17.1	.76
	4	8.77	52.2	27.0	42.9	28.0	7.46	20.5	.73

* Surface area in square meters $= 0.112 \sqrt[3]{(\text{body wt in kg})^2}$ (Lusk, G., The Science of Nutrition, W. B. Saunders Co., pp. 122-123).

the formation of a necrotic lesion of the skin, the second, on the other hand, did not produce macroscopically visible local lesions.

Nothing definitive can be said about the possible mechanism of the local resistance observed. It is quite possible that the inflammatory state provoked by the formalin diminished the absorption of the lethal dose.[7,8,9]

Although local resistance is not frequently encountered, it is not a new phenomenon. Rosenthal, Tabor, and Lillie observed that mice surviving tourniquet shock because of salt treatment would survive a repetition of the same shock without salt, if given in the same leg, but would die if it was given in a different leg.[10] Similarly, according to a review by J. Levy, animals in which resistance to arsenic has been built up *per os* do not tolerate a toxic dose administered subcutaneously.[11]

Summary. When mice were pretreated with adrenalin they showed general resistance to a lethal dose of this substance but not cross resistance to formalin, either local or general. On the contrary, animals pretreated with formalin revealed only a local resistance, whether they were subjected to a lethal dose of formalin or of adrenalin. These observations seem to question Selye's interpretation of some of his experiments on general adaptation to the same or different "alarming" stimuli.

7 Favilli, Giovanni, and McClean, D., *J. Path. and Bact.*, 1937, 45, 661.

8 Duran-Reynals, F., *Bact. Rev.*, 1942, 6, 197.

9 Menkin, Valy, *Dynamics of Inflammation*, New York, The Macmillan Co., 1940, p. 165.

10 Rosenthal, S. M., Tabor, Herbert, and Lillie, R. D., *Am. J. Physiol.*, 1945, 143, 402.

11 Levy, Jeanne, *Bull. de la Soc. de Chimie Biologique*, 1934, 16, 631.

16165

Renal Reabsorption of Methionine in Normal Dogs.*

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It is known that at normal plasma concentrations, reabsorption of amino acids from the glomerular filtrate is practically complete. Experiments in which the plasma concentration has been raised above normal levels have shown, however, that there are differences in the efficiency with which individual amino acids are reabsorbed. These differences are manifested in the rates of reabsorption of the various acids prior to the attainment of the maximal rate (Tm), and also in the levels at which the Tm is reached.[1-5]

The experiments to be described were designed to study the renal reabsorption of methionine. Shortly after they were concluded, experiments utilizing the microbiological method of analysis were reported[5] which indicated that the reabsorption of methionine is practically complete at plasma levels up to 115 mg % (equivalent to about 10.8 mg

1 Pitts, R. F., *Am. J. Physiol.*, 1943, 140, 156.

2 Pitts, R. F., *Am. J. Physiol.*, 1944, 140, 535.

3 Ferguson, F. P., Byer, F. T., and Eaton, A. G., *Fed. Proc.*, 1945, 4, 20.

4 Eaton, A. G., Ferguson, F. P., and Byer, F. T., *Am. J. Physiol.*, 1946, 145, 491.

5 Wright, L. D., Russo, H. F., Skeggs, H. R., Patch, E. A., and Byer, K. H., *Am. J. Physiol.*, 1947, 149, 130.
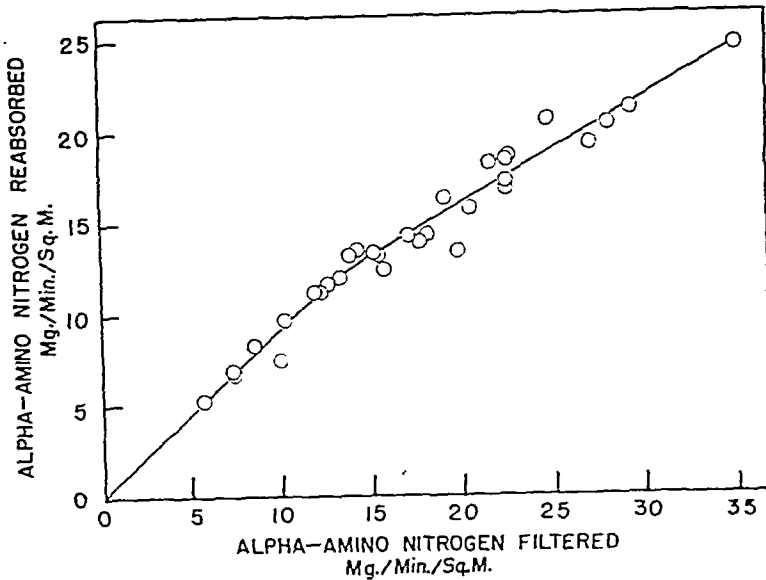
Fig. 1.

The relation between renal filtration and reabsorption of alpha-amino nitrogen of dl-methionine in Dog III.

which are considerably higher than the maximal rates reported for leucine, isoleucine and valine[1] and which stand in strong contrast to such low threshold amino acids as arginine and lysine.[3,5]

Summary. The relation between the rate of renal filtration and reabsorption of dl-methionine α-amino nitrogen has been studied in normal dogs. As the amount filtered was increased to 34.8 mg α-amino nitrogen/min/square meter, the highest level attained in these experiments, the rate of reabsorption increased to 24.8 mg/min/square meter and there was no evidence that a Tm value was obtained. The data demonstrate the high efficiency of the renal mechanism for the reabsorption of methionine.

16166 P

Cataracts Resulting from a Deficiency of Phenylalanine in the Rat.*

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Experimental cataracts in the rat have been shown to result from deficiencies of either of 2 of the essential amino acids. What apparently were cataracts due to tryptophane deficiency in the rat were first described by Curtis, Hauge and Kraybill.[1] Totter and Day[2] later made a careful study of the cataracts resulting from this deficiency. Recently,

[1] Curtis, P. B., Hauge, S. M., and Kraybill, H. R., *J. Nutrition*, 1932, 5, 503.

[2] Totter, J. R., and Day, P. L., *J. Nutrition*, 1942, 24, 159.

% methionine α-amino nitrogen). The results of the present study, based upon α-amino nitrogen determinations and extending the plasma concentrations beyond the levels previously reported, provide further evidence for the high efficiency of the renal reabsorptive process for methionine.

Methods. The experiments were carried out upon 2 healthy female dogs in the manner described in detail elsewhere.[4] Infusions of appropriate concentrations of inulin and dl-methionine in physiological saline were made into the femoral vein at a rate of about 5 cc per minute. Urine collections were made from the bladder with an indwelling catheter and blood samples were taken from the femoral artery by the use of an indwelling spinal needle cut to a length of 4 cm and equipped with a tightly fitting stylet. Periods were 30 minutes in duration. Blood samples of 10 cc were taken at the beginning, mid-point and end of each period and the average amino acid and inulin concentration of the 3 samples employed to indicate the level for the period.

The plasma amino acid concentration was determined manometrically by the ninhydrin-carbon dioxide method of Hamilton and Van Slyke[6] and urinary amino acid was similarly determined by the method of Van Slyke, MacFadyen and Hamilton.[7] Inulin was determined by the method of Hubbard and Loomis[8][§] using a Coleman spectrophotometer to measure the intensity of color developed.

Results. Eight different experiments, involving 31 periods, were performed upon one dog and 3 experiments involving 8 periods on another. Plasma levels up to 27 mg % of α-amino nitrogen and filtration rates up to 34.8 mg per minute per square meter of body

surface were obtained. In all of the experiments, the efficiency of the renal tubules in reabsorbing methionine from the glomerular filtrate proved to be very high. There was no indication that the maximal rate of reabsorption was reached at any of the levels of glomerular filtration which were attained.

Table I shows the essential data obtained in four of the experiments performed upon Dog III. Each of these experiments was repeated upon the dog with similar results. Inulin clearance has been used as a measure of glomerular filtration rate[9] and the rates of filtration, excretion and reabsorption of α-amino nitrogen have been calculated as previously described.[4] It is seen that as the filtration of amino nitrogen increases through 14 mg/min/square meter reabsorption is well over 90% complete. Beyond this level, the proportion reabsorbed decreased slightly for this dog, but remained remarkably high throughout the experiments. In the experiments upon the second dog (No. IV), plasma α-amino nitrogen concentrations up to 19.8 mg % and filtration rates up to 32.5 mg/min/square meter were attained. Except for the fact that the ratio of reabsorbed : filtered amino nitrogen remained above 0.82, even at the highest filtration levels, the results were in all respects similar to those described for Dog III.

Fig. 1 shows graphically the relationship between the amount of methionine α-amino nitrogen filtered and that reabsorbed in all eight of the experiments performed upon Dog III. It will be noted that although the slope of the curve decreases somewhat beyond filtration rates of around 14 mg/min/square meter, there is no tendency to plateau even at the highest levels attained.

From these experiments it is evident that the tubular capacity for the reabsorption of methionine is relatively high. In terms of α-amino nitrogen it exceeds 25 mg/min/square meter. In this respect, methionine resembles glycine[1] and alanine.[2] These substances manifest reabsorption capacities

6 Hamilton, P. B., and Van Slyke, D. D., *J. Biol. Chem.*, 1943, 150, 231.

7 Van Slyke, D. D., MacFadyen, D. A., and Hamilton, P. B., *J. Biol. Chem.*, 1943, 150, 251.

8 Hubbard, R. S., and Loomis, T. A., *J. Biol. Chem.*, 1942, 145, 641.

§ This method was modified to the slight extent of heating the color-forming solution at 80°C for 14 minutes instead of 8 as prescribed by the authors. This modification assured the maximal development and stability of color.

9 Smith, H. W., *The Physiology of the Kidney*, Oxford University Press, 1937.

liver stimulants.

The constituent chiefly responsible for the drastic laxative action of podophyllin is podophyllotoxin.[1] This is a highly toxic compound which causes mucosal inflammation and gastroenteritis. It likewise involves the nervous system resulting (in the cat) in disturbance of coordination of the posterior extremities, rapidly increasing weakness, increased respiratory rate, violent colonic cramps and death in coma.[2,3] The parenteral administration of podophyllin to chickens likewise resulted in nervous system involvement and cytological examination revealed damage or complete degeneration of the cerebellar Purkinje cells and other nervous elements.[4]

Kaplan[5] demonstrated that a suspension of podophyllin in oil was highly effective against *Condylomata acuminata*. These results were confirmed and extended by King and Sullivan[6,7] and by Sullivan and Blanchard.[8] These investigators likewise noted the similarity of podophyllin to colchicine in its effects on venereal warts. Sullivan and Wechsler[9] have shown that saturated aqueous solutions of podophyllin block mitosis in the root tips of *Allium cepa*. In very dilute solutions both podophyllin and podophyllotoxin destroy the mitotic spindles of cleaving *Asterias* and *Arbacia* eggs, as discovered recently by Cornman.[10]

The effects of podophyllin on *Condylomata acuminata* and *Allium* made this compound

of interest to us as a possible therapeutic agent in the treatment of cancer. Our interest was increased by the incidental use in tissue culture of placental serum from a patient previously treated with podophyllin for *Condylomata acuminata*. This placental serum caused severe damage to mouse tumor cells growing in roller tubes without affecting normal cells growing in the same tubes.

Material and Methods. It was considered desirable to design a method for the detection of materials of possible value in the chemotherapy of cancer which take advantage of the tissue culture techniques for mammalian cells. Since we planned to test a large number of materials it was necessary that the test be one which gave a maximum of information within a short time. The roller tube technique of Gey and Gey[11] was consequently adopted. In this procedure thin-walled pyrex glass tubes (150 mm x 15 mm) are used. Each tube contains 6 pieces of normal embryonic mouse skin arranged in a row along the bottom third of the tube and a row of 6 mouse tumor fragments similarly arranged on the opposite wall of the tube. Occasionally a third row of 6 pieces of a second tumor is placed in the tube. The mouse tumors used have been in-strain transmittable mouse sarcoma L946 from C-57 mice and lung tumor MA387 from AK mice.

The tissue fragments are held in place with a thin layer of chicken plasma clot. The nutrient medium totaling one ml consists of 0.4 ml Gey's solution, 0.2 ml chick embryo extract, 0.1 ml human placental serum and 0.3 ml horse serum. The tubes are closed with sterile rubber stoppers and incubated at 37°C in a rotating drum.

Our customary procedure is to incubate the tubes for 24 hours, then examine and grade the cells that have grown or migrated from each explant. Nutrient medium to the volume of 0.1 ml is withdrawn from the tube and the test material in a volume of 0.1 ml is then added to the nutrient medium. The tubes are incubated for another 24 hours, and the growing cells again graded.

1 Magnus, R., *Handbuch der Experimentellen Pharmacologie*, ed. A. Heffter, vol. 2, pt. 2, p. 1645, J. Springer, Berlin, 1924.

2 Viehover, A., and Mack, H., *J. Am. Pharm. Assn.*, 1938, 27, 632.

3 Chenoweth, M. B., Hunt, C. C., and Philips, F. S., 1947, personal communication.

4 MacCardle, R. C., and Perrault, A., *Fourth Int. Cancer Cong.*, 1947, abstract.

5 Kaplan, I. W., *New Orleans Med. and Surg. J.*, 1942, 94, 388.

6 King, L. S., and Sullivan, M., *Science*, 1946, 104, 244.

7 Sullivan, M., and King, L. S., *Arch. Derm. Syph.*, 1947, 56, 30.

8 Sullivan, M., and Blanchard, D., *Bull. Johns Hopkins Hosp.*, 1947, 81, 65.

9 Sullivan, B. J., and Wechsler, H. I., *Science*, 1947, 105, 433.

10 Cornman, I., *Biol. Bull.*, 1947, in press.

11 Gey, G. O., and Gey, M. K., *Am. J. Cancer*, 1936, 27, 45.

Sydenstricker, Schmidt and Hall[3] have reported the development of cataracts in rats on a histidine-deficient diet. The study reported here was occasioned by the discovery of a well-developed cataract in a rat fed on a diet deficient in phenylalanine.

Methods. The rats used were from a Wistar strain. When 25-28 days of age they were placed in individual cages and given experimental diet and water *ad libitum*. The phenylalanine-deficient diet and a similar control diet used were those described by Sydenstricker, Hall, Bowles and Schmidt.[4] The development of cataracts was studied in 15 rats from 5 litters which were fed the phenylalanine-deficient diet. Seven rats from the same litters were fed the control diet. Three times weekly the eyes were examined for lenticular abnormalities with the biomicroscope after dilating the pupil with 0.5% solution of atropine sulphate in physiological saline solution. As soon as possible after the death of each deficient rat the lenses were removed, immersed in saline, and examined under a dissecting microscope.

Three deficient rats with cataracts of varying degrees of development were changed to the control diet so that any resulting regression of the lenticular changes could be observed.

Results. Definite lenticular changes were observed in all but 2 of the 15 rats in from 17 to 33 days (mean, 22 days). These changes continued to develop, resulting in a progressive lessening of the transparency of the lenses until at the time of death, after 20-53 days on the diet, various degrees of opacity were observed. The earliest change was a slight haziness of the lens substance. Next the lens star became visible. This was followed by a separation of the superficial fibers, and sometimes by a rough granular appearance of the epithelial layer. No changes in the lens capsule were observed. Finally, in the posterior portion of the lens a dense central opacity developed, eventually filling the central portion of the lens and leaving only a narrow peripheral portion comparatively clear.

Changing the deficient rats to the control diet resulted in a reversal of only the more superficial changes in the lens.

In none of the control rats were any lenticular changes observed.

Summary. The lenticular changes observed to result from phenylalanine-deficiency in the rat were diffuse haziness, opacity of the star, progressive separation of the fibers, granular changes in the epithelial layer and the development of dense central cataracts. Only the more superficial changes in the lenses were found to be reversed by adding *dl*-phenylalanine to the diet.

3 Sydenstricker, V. P., Schmidt, H. L., Jr., and Hall, W. K., PROC. SOC. EXP. BIOL. AND MED., 1947, 64, 59.

4 Sydenstricker, V. P., Hall, W. K., Bowles, L. L., and Schmidt, H. L., Jr., *J. Nutrition*, 1947, 34, 481.

<div align="center">16167</div>

Effect of Podophyllin on Tumor Cells in Tissue Culture.*

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Podophyllin, N.F. is a mixture of substances derived from the roots and rhizomes of the mandrake plant by hot ethanol extraction and subsequent precipitation in dilute HCl. Various preparations of the mandrake plant have been in use for at least a hundred years as laxatives and more recently as supposed

TABLE I.

Screening Test—Podophyllin, 12 mg/l; N, Normal Fetal Mouse Skin; L, Sarcoma, L946; M, Lung Tumor MA387.

	Tube No. 1 24-hr data			Tube No. 2		
	N	L	M	N	L	M
Growth	12	22	16	18	29	15
Lysis inhibition	24	21	20	24	22	17
% rounding	0	1	0	0	1	0
Degree rounding	0	3	0	0	3	0
% granulation	24	24	24	24	24	24
Degree granulation	12	12	12	12	12	12
Disintegration	0	0	0	0	0	0
Total	36	40	36	36	40	36
	48 hr data					
Growth	14	25	19	18	24	19
Lysis inhibition	24	14	19	24	18	16
% rounding	0	24	20	0	24	20
Degree rounding	0	12	9	0	18	11
% granulation	24	24	24	24	24	24
Degree granulation	12	18	17	12	18	18
Disintegration	0	0	1	0	0	0
Total	36	78	71	36	84	73

Selective index of L946 = 41.
Selective index of MA387 = 36.

details of nuclear structure can be seen in the living cells, stained preparations indicate considerable nuclear derangement. The chromatin is represented by disorganized fragments in most cases and considerable degree of pycnosis in others. A slight amount of cellular disintegration often is manifest by the presence of cellular debris in the culture. Essentially the same phenomena occur in normal cells but only at higher concentrations of podophyllin.

Selective damage to the tumor cells as compared with normal cells is obtained with podophyllin over a concentration range of 0.08-20.0 mg 1. Within these concentrations the Selective Index varies from 10 to 45, with the highest scores occurring in the concentration range of 0.3-2.5 mg/1.

This picture of cellular damage and destruction is reversible under some conditions. When podophyllin concentrations of 5 mg/1 or less are employed, and the podophyllin removed after 24 hours' treatment by replacement with fresh normal medium, the normal cells may recover normal appearance and resume normal growth. Tumor cells, on the other hand, have not shown recovery unless the concen-

trations employed were 0.6 mg/1 or below.

This compares with results found with crude penicillin, from which normal fibroblasts quickly recovered whereas sarcoma cells died.[12]

The specific effects of podophyllin and its components on karyokinesis and cell division are of considerable interest, particularly in view of the fact that nuclear damage including abnormal mitosis has been produced in sarcoma L946 and lung tumor MA387 growing in mice. In these experiments extensive damage to mouse tumors in vivo was produced by the parenteral administration of podophyllin. Studies on cell division as well as investigations of the biochemical and carcinoclastic actions of this material are now in progress.

Tissue culture tests with podophyllotoxin indicate that this compound is not as effective as crude podophyllin in causing selective damage to tumor cells. Also, the concentration range within which these slight effects are obtained is quite narrow, in contrast to the wide effective concentration range of podo-

12 Cornman, I., J. Gen. Physiol., 1944, 28, 113.

The tumor tissue is removed at this time and implanted into susceptible mice. The normal tissue is grown for 4 days longer in fresh normal nutrient medium and again graded.

In grading the outgrowths of cells from the original tissue fragments at 24 hours, 48 hours, and again at 6 days, all visible microscopic changes that can be adequately observed in living cells are examined. The following points are noted and a grade of 0 to 4 assigned to all except "growth" for which the score runs from 0 to 6; 1) "growth," 2) inhibition of lysis of the plasma clot, 3) number of abnormally rounded cells, 4) severity of cell rounding, 5) number of cells with granulated cytoplasm, 6) intensity of cytoplasmic granulation, and 7) extent of cell disintegration.

The scores for 3, 4, 5, 6, and 7 are added and by subtracting similarly calculated control values, a damage score for tumor cells termed the "Selective Index" is derived. This is represented by the formula—

$$SI = \left[(T^E_{48} - T^E_{24}) - (N^E_{48} - N^E_{24}) \right] - \left[(T^C_{48} - T^C_{24}) - (N^C_{48} - N^C_{24}) \right]$$

where T represents tumor tissue, N represents normal embryonic mouse skin, the superscript E represents experimental tube, the superscript C represents control tube and the subscripts 48 and 24 refer to the composite damage grades at those hours.

The Selective Index plus the scores for "growth" and inhibition of lysis of the plasma clot are useful in assessing the effects of chemotherapeutic candidates. The comparative effects on normal and tumor tissue of a large series of materials can be easily compared on this basis. The term "growth" here means both cell migration and cell proliferation and actually represents simply the increase around the original explant in the area which is covered by cells originating in the explant.

Selective Indices of 0 to 10 are considered negative, 10 to 30 are considered significant and over 30 highly significant. Less than 10% of all materials thus far tested in this manner have given significant differential effects. A number of substances that have been used more or less extensively in the treatment of human cancer have given negative results in this test. These include various nitrogen mustards, heptaldehyde, urethane, Fowler's solution, benzene and stilbamidine.

The podophyllin in these experiments was precipitated from a 95% ethanol solution in 10% gum acacia and subsequently diluted in 0.87% NaCl to the desired concentrations.

Experimental. Table I shows the results of a representative experiment with podophyllin. In this experiment each tube contains 6 pieces each of normal fetal skin, L946 and MA387. The figures presented each represent the sum of the scores for 6 tissue fragments. In this experiment the terms $(T^{48}_C - T^{24}_C)$ and $(N^{48}_C - N^{24}_C)$ were zero in parallel control preparations and $(N^{48}_E - N^{24}_F)$ can be seen to be zero from Table I. The Selective Index was calculated therefore simply by subtracting the 24-hour tumor damage scores from the 48-hour tumor damage scores and averaging the results from the 2 tubes.

It is seen that podophyllin is significantly more damaging *in vitro* to mouse tumors L946 and MA387 than to normal embryonic mouse tissue including fibroblastic and epithelial elements.

Under the influence of podophyllin the tumor cells, which are typically spindle shaped and have long slender cytoplasmic processes, become rounded or lobose in form. Migration of the cells from the explant appears to be repressed. Normal fibroblast cells show the same changes but only at much higher concentrations of podophyllin. The tumor cells (unstained) which normally spread out in a loose interlacing sheet, coalesce into a dense and closely packed sheet or ring around the original explant. Isolated cells in the plasma clot appear rounded and smaller, as though some dehydration had occurred. The nucleus disappears from sight leaving only granular cytoplasm. There is a striking change in the normally fine cytoplasmic granulation. The fine granules are replaced by larger granules which aggregate in clumps throughout the cytoplasm. The non-granular portion of the cytoplasm loses its fine structure and assumes a clear hyaline appearance. Although few

TABLE I.

The Effect of Varying the Aqueous Humor/Chicken Serum Ratio in the Supernatant of Roller-Tube Cultures of Sarcoma 180. Data represent average of 2 or more experiments. A.H. = Aqueous Humor; C.P. = Chicken Plasma; C.S. = Chicken Serum; 37°C.

Clot	Supernatant concentration of AH to C.S.	Lysis		Growth	
		24 hr	72 hr	24 hr	72 hr
AH/CP = 1:2	3:1	0	2.0	2.5	4.5
,,	2:1	0	1.0	2.0	4.5
,,	1:1	0	0	3.0	4.5
,,	1:2	0	0	1.5	4.0
,,	1:3	0	0	2.5	4.5

TABLE II.

Growth of Sarcoma 180 in the Absence of Serum. Data from one experiment but are representative of additional duplicate trials. 37°C.

Clot	Supernatant (drops)		Lysis 48 hr	Growth 48 hr
	Salt solution	Chicken embryo extract		
Chicken plasma	10	0	0	3.5
,, ,,	9	1	0	4.5
,, ,,	8	2	0	4.0
,, ,,	7	3	0	4.5
,, ,,	6	4	0	4.5

faction although they have been unsuccessful in extracting such a component from tissue.

In our experiments, mouse Sarcoma 180 was grown in roller tubes in a chicken plasma clot with a supernatant ordinarily composed of a balanced salt solution,[6] mammalian serum and chicken embryo extract (one part minced whole embryo to one part balanced salt solution). In such a preparation the clot was partly liquefied within 24-48 hours. This liquefaction sometimes continued until the tissue fragment was entirely surrounded by a ring of liquefaction. Our investigation was concerned with attempts to control such liquefaction and to examine the nature of the mechanism involved by altering the medium in which the tumor is customarily grown.

Material and Methods. The ability of various tumors to grow in the anterior chamber of the rabbit's eye suggested the use of aqueous humor in tissue culture. In our first experiments, the aqueous humors of rabbit, sheep or steer were incorporated in the plasma clot. The effect of modifying the serum content of the supernatant nutrient medium was also examined. Preliminary experiments were

carried out in Maximow slides in which hanging drop cultures were set up with various ratios of aqueous humor and chicken plasma in the clot. Subsequent experiments were carried out in roller tube cultures following the technique of Gey and Gey.[6] Tissue fragments were oriented in glass culture tubes and then covered with a layer of chicken plasma. After clotting of the plasma was complete, the supernatant fluid was added and the tubes were incubated at 37°C in a rotor. Seven to 12-day-old mouse Sarcoma 180 tumors, carried in CFW mice, were used in all the experiments. The degree of lysis was graded by dividing the perimeter of the tumor fragment into four quadrants, each with a value of one. Thus, lysis in one quadrant was assigned a grade of 1; 2 quadrants a grade of 2, etc. The amount of growth was graded in a similar manner. A grade of 4 indicated a complete fringe of growth around the fragment. When the width of growth was equal to the diameter of the fragment, a grade of 5 was assigned.

Results. In Maximow slide hanging drop preparations in which aqueous humor was incorporated in chicken plasma clots no lysis of the clot appeared over a 4-day period, al-

[6] Gey, G. O., and Gey, M. K., *Am. J. Cancer*, 1936, 27, 45.

phyllin.

Conclusions. 1. Podophyllin exerts a selective damaging effect on mouse tumor cells in tissue culture over the concentration range 0.08-20.0 mg/l. 2. This damaging effect is more easily reversible in normal than in tumor cells. 3. Podophyllotoxin is not as effective as podophyllin in causing selective tumor damage. 4. *In vivo* studies with tumor-bearing mice confirm the selective tumor damaging effects of podophyllin which were first noted in tissue culture preparations.

16168

Experimental Alteration of the Ability of Tumor Cells to Lyse Plasma Clots *in vitro.*[*]

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One of the outstanding characteristics of tumor cells when grown in tissue culture is their ability to lyse the plasma clot in which they are embedded. This liquefaction of the clot has been mentioned by many investigators. Drew,[1] comparing cultures of normal and malignant tissues *in vitro*, noted that a 24-hour culture of mouse embryo heart tissue showed a ring of growth around the periphery of the original fragment, while a corresponding culture of mouse sarcoma showed a similar ring of growth separated from the original explant by a circular area of liquefaction. The growth of mouse sarcoma in rat plasma was described by Lambert and Hanes[2] as being "ring-form," since lysis of the clot allowed the contracting fibrin to retract from the original fragment, leaving the fragment situated on the periphery of the circle of cells like the setting in a signet ring. Carrel and Burrows[3] attributed their lack of success in the cultivation of human carcin-

oma to rapid liquefaction of the plasma clot.

It is important in the analysis of the nature of this lytic process to note that lysis of a plasma clot can be induced by normal tissue. Over 30 years ago, Fleisher and Loeb[4] examined the fibrinolytic effect of various tissue fragments surviving *in vitro* on the plasma of different animals. In general, it was found that mammalian tissues lysed clots formed from mammalian plasma, but had no lytic effect on chicken plasma clots. It was further reported that normal chicken tissues were completely ineffective in producing lysis of either mammalian or chicken plasma clots. The liquefying power of tissues usually decreased when a large amount of chicken plasma was added to the standard rabbit plasma clot.

Very recently, Astrup and Permin[5] reported that tissue slices from different mammals (ox, pig, rabbit and rat) produced lysis of the ox fibrin clots in which they were embedded, the degree of lysis depending on the organ and species of animal used. They, too, found that chicken tissues did not cause lysis of such clots. They suggested that a pro-fibrinolysin in the plasma is activated by a cellular kinase, and thereupon causes lique-

1 Drew, A. H., *Brit. J. Exp. Path.*, 1922, **3**, 20.

2 Lambert, R. A., and Hanes, F. M., *J. Exp. Med.*, 1911, **13**, 495.

3 Carrel, A., and Burrows, M. T., *J. Exp. Med.*, 1911, **13**, 571.

4 Fleisher, M. S., and Loeb, L., *J. Biol. Chem.*, 1915, **21**, 477.

5 Astrup, T., and Permin, P. M., *Nature*, 1947, **159**, 681.
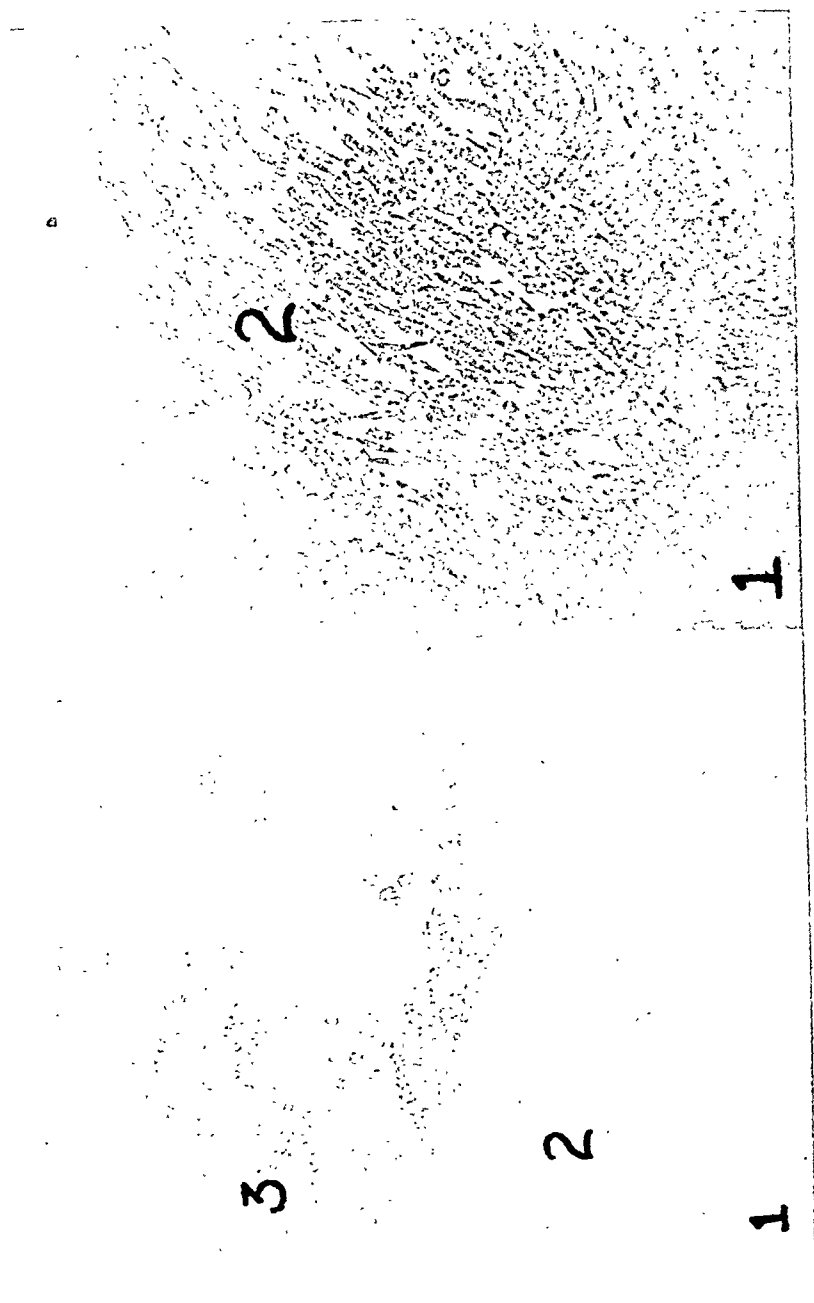
FIG. 1.

72 hour growth of Sarcoma 180 using unheated and heated human serum in the supernatant. Living cultures in roller tubes, × 80.
A—unheated human serum; 1—original explant; 2—lysed area; 3—moribund cells.
B—heated human serum; 1—original explant; 2—extensive outgrowth of Sarcoma 180 cells.

activation of the serum containing profibrinolysin or the omission of serum from the culture medium explains the lack of lytic activity in such cases. The different degrees of lysis occurring when various sera are used may be due to differences in the amount of profibrinolysin present in the different sera. Correspondingly, mammalian aqueous humor can be considered as a dilute mammalian serum which contains only profibrinolysin.

though there was extensive tissue growth. The most vigorous growth was obtained with concentrations of aqueous humor to chicken plasma of 1:1 and 1:2. Normal growth of the tissue for a period exceeding 4 days has not been successful, the cells becoming extremely granular and rounded, and eventually disintegrating.

All subsequent experiments were carried out in roller tubes. In these preparations lysis of the aqueous humor/chicken plasma clots occurred when a supernatant fluid containing mammalian serum was present. By varying the constituents of the supernatant, it was possible to block this lytic action. If the ratio of aqueous humor to chicken serum was 1:1 or 1:2 in the supernatant fluid. no lysis occurred. Further experiments (Table I) showed that the chicken serum was the responsible factor in preventing lysis. Aqueous humor did not inhibit lysis. On the contrary, if present in high enough concentration, it promoted fibrinolysis.

Table I shows that as the concentration of aqueous humor decreased and the concentration of chicken serum increased, the amount of lysis decreased. At an aqueous humor/chicken serum ratio of 1:1 in the supernatant, lysis was completely absent and growth was apparently unaffected. With higher concentrations of chicken serum in the supernatant, lysis was similarly absent although growth appeared to be adversely affected.

When all sera were omitted from the supernatant fluid, leaving only the balanced salt solution and chicken embryo extract, no lysis of the chicken plasma clot occurred, although there was good growth. The addition of chicken embryo extract did not stimulate lytic activity even though more extensive growth occurred. These data are presented in Table II.

Table III summarizes the roller tube experiments in which the supernatant medium was altered in attempts to prevent lysis of the clot. In these experiments a clot composed solely of chicken plasma was employed.

It will be noted that the presence of avian serum in the supernatants is correlated with the occurrence of little or no lysis, while the

TABLE III.

Plasma Clot Lysis at 48 Hours in Roller Tube Cultures of Mouse Sarcoma 180 Cells in the Presence of Various Sera. 37°C. C.P. = Chicken plasma; H.S. = Horse serum; C.S. = Chicken serum; R.S. = Rabbit serum; Hu.S. = Human serum.

Clot	Supernatant*	Lysis	Growth
C.P.	Chicken serum	0	4.5
,,	Duck serum	0	4.5
,,	Horse serum	2.0	5.0
,,	H.S. + C.S. (2:3)	0	5.0
,,	Rabbit serum	2.0	4.5
,,	R.S. + C.S. (2:3)	2.0	5.0
,,	Human serum	4.0	4.5
,,	Hu.S. + C.S. (2:3)	2.5	4.5
	Hu.S. + C.S. (2:4)	1.0	4.5

* Includes a balanced salt solution and chicken embryo extract.

presence of mammalian sera resulted in a significant degree of lysis. The addition of sufficient amounts of chicken serum (replacing an equal volume of the balanced salt solution) to the mammalian sera resulted in loss of lytic activity, rabbit serum being an exception—at least at the concentrations tested.

With human serum in the supernatant, the liquefaction at 72 hours was so complete around the fragment that it was occasionally washed away, leaving an empty space surrounded by a ring of cells. The use of serum previously heated at 56°C for 3 hours resulted in the complete absence of lysis and rapid growth of the cultures. Fig. 1 shows the effect of heating the serum.

Discussion. Inasmuch as omitting serum, using heterologous serum, or heating the serum affects the amount of liquefaction, it seems probable that serum contains a factor essential to the lytic mechanism. Since the area of liquefaction is invariably contiguous with the tumor fragment, it appears that the tissue also contributes to the lytic mechanism. The experimental data presented are in harmony with blood enzyme studies[7] in which it has been suggested that an active proteolytic enzyme is produced as the result of an interaction of a cell activator (streptokinase) and the profibrinolysin normally present in plasma and serum. According to this theory heat in-

[7] Christensen, L. R., *J. Gen. Physiol.*, 1945, **28**, 363.

that there was no lysis.

The anti-lytic effect of chicken plasma used in culturing mammalian tumors has been established by other investigators. Gey and Gey[6] have reported that the use of a large amount of chicken plasma helped to prevent early liquefaction, but sometimes delayed growth. A strain of sarcoma cells derived from a dibenzanthracene mouse tumor was cultured *in vitro* for more than two years by Jacoby,[10] with almost no liquefaction of the chicken plasma clot. The supernatant consisted of chicken serum, chicken embryo juice and Tyrode solution. Lewis and Strong,[11] in a survey of over 50 different spontaneous mouse tumors in tissue culture, found that the cultures rapidly liquefied the clot when it was composed of mouse plasma or a mixture of mouse and chicken plasma. In chicken plasma, however, such liquefaction did not occur.

In assigning the profibrinolysis to the serum and the kinase to the cells we are following the current ideas of Christensen and McLeod.[7,12] Our data are not such as to eliminate the inverse possibility: profibrinolysin in the tissue activated by a blood kinase.

10 Jacoby, F., *Nature*, 1943, 152, 299.

11 Lewis, M. R., and Strong, L. C., *Am. J. Cancer*, 1934, 20, 72.

12 Christensen, L. R., and McLeod, C. M., *J. Gen. Physiol.*, 1945, 28, 559.

We cannot assume that there are differences only in the amount of profibrinolysin of various animal sera. There may be differences in the amounts of activator released by the cells of animals of different orders, since Fleisher and Loeb,[4] and Astrup and Permin[5] have reported differences in the lytic activity of tissues from various animals.

Summary. 1. Mouse Sarcoma 180 cells, in the presence of mammalian serum, cause lysis of chicken plasma clots. 2. Mammalian aqueous humor has slight influence on the fibrinolytic process. 3. When Sarcoma 180 is grown in the presence of avian serum, no lysis occurs. If a sufficient quantity of chicken serum is added to a supernatant fluid containing mammalian serum there is a decrease in the amount of clot lysis. 4. Lysis of the plasma clot by Sarcoma 180 does not occur when mammalian serum previously heated to 56°C for 3 hours is used, or when all serum is omitted from the supernatant. 5. The different degrees of lysis obtained when sera from various mammalian orders are used may be due to differences in the amount of profibrinolysin contained in the various sera. 6. There are two factors necessary for the lytic mechanism, tentatively classified as a profibrinolysin from the serum, and an activator of the profibrinolysin derived from the tissue.

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Dichotomy Between Hypophyseal Content and Amount of Circulating Gonadotrophins During Starvation.[*]

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The amounts of gonadotrophin contained within the pituitary gland have often been assumed to parallel the amount produced and released into the blood stream. Since the gonadotrophic hormone content of the hypo-physis must be the resultant of the amounts produced and the amounts released, they do not necessarily parallel each other. It is the purpose of this investigation to assess the effect of acute starvation on pituitary gonadotrophic hormone content and its release into the circulation.

The effective amount of circulating gonad-

When mammalian Sarcoma 180 is grown in the presence of avian serum, the absence of lysis in this case may be due to (1) lack of sufficient profibrinolysin in the serum, or (2) the inability of mammalian tissue to activate avian profibrinolysin. Since the Rous chicken sarcoma causes extensive liquefaction of chicken plasma clots,[8] it appears that chicken plasma does contain profibrinolysin. It is more likely, therefore, that mammalian tissue is unable to activate avian profibrinolysin. The inhibitory effect of chicken serum on the lytic activity of various mammalian sera may be explained by assuming that the profibrinolysin of the chicken serum combines with the activator from the mammalian tumor Sarcoma 180 to form an inactive complex:

Kinase   + Profibrinolysin ⟶ Fibrinolysin
(Mammalian   (Mammalian   (Active)
  tissue)   serum)
Kinase   +Profibrinolysin ⟶ Kinase·
(Mammalian   (Avian serum)   Profibrinolysin
  tissue)   (Inactive)
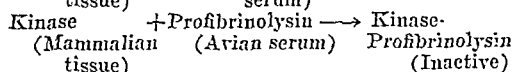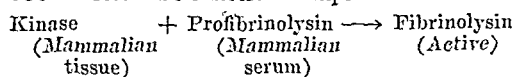
Thus, if sufficient avian serum is added to mammalian serum in the presence of mammalian cells, it effectively removes the mammalian kinase through formation of the inactive complex and prevents activation of the mammalian profibrinolysin and subsequent lysis of the fibrin.

This hypothesis is further supported by the data presented in Table IV. Chicken serum previously heated to 56°C for 3 hours was added to a mammalian tissue preparation with a supernatant containing mammalian serum. No inhibition of the lytic process was observed.

The absence of lysis in preparations in which heated human serum was used exclusively in the supernatant might be explained by postulating the activation of inhibitory substances due to prolonged heating at 56°C. However, the addition of heated human serum to supernatants containing only normal human serum did not result in any significant effect on the lytic process. This indicates that the heated human serum did not contain an inhibitor, and that the absence of lysis in tubes in which the supernatant contained only heated serum is best explained on the basis of de-

8 Fisher, A., *Nature*, 1946, **157**, 442.

TABLE IV.

Effect of Heated Serum on Lysis in the Presence of Unheated Human Serum. Each datum represents duplicate determinations. C.P. = Chicken plasma; C.S. = Chicken serum; Hu.S. = Human serum. Roller tube cultures, 37°C.

Clot	Supernatant*	48 hr Lysis	Growth
C.P.	Hu.S. + C.S. (2:4)	1.0	4.5
,,	,, + heated C.S. (2:4)	3.5	4.5
,,	,, + (2)	3.5	4.0
,,	,, + (6)	3.5	4.0
,,	,, + heated Hu.S. (2:2)	4.0	4.0
,,	,, + ,, ,, (2:4)	3.0	4.0

* Includes a balanced salt solution and chicken embryo extract.

struction of a proenzyme or enzyme concerned with fibrinolysis.

Fischer,[8] working with the Rous chicken sarcoma, which liquefies chicken plasma clot, was able to grow this tumor without liquefaction in a clot made from rabbit plasma. In this case it may be that the reciprocal event occurs: avian tissue kinase forms an inactive complex with mammalian profibrinolysin. Fischer further found that on the addition of fresh chicken serum to the rabbit plasma, lysis of the clot occurred; whereas chicken serum heated at 56°C for 3-4 hours produced no such action. Since chicken tumor cells caused liquefaction of the clot in the presence of chicken plasma or chicken serum but not in the presence of rabbit plasma, Fischer concluded that lysis occurred only in the presence of homologous plasma or serum. It is not clear how exclusive his use of the term "homologous" is intended to be. We have shown that differences in sera from other mammalian orders are not sufficient to prevent lysis by mouse Sarcoma 180. Lambert and Hanes[9] demonstrated that when rat and mouse sarcoma cells were grown in the plasma of various animals, liquefaction occurred in the presence of the plasma of guinea pig, rabbit, dog and human (the latter producing the greatest amount of liquefaction). In goat plasma there was neither growth nor lysis. Although there was good growth in pigeon plasma, the authors imply

9 Lambert, R. A., and Hanes, F. M., *J. Exp. Med.*, 1911, **14**, 129.
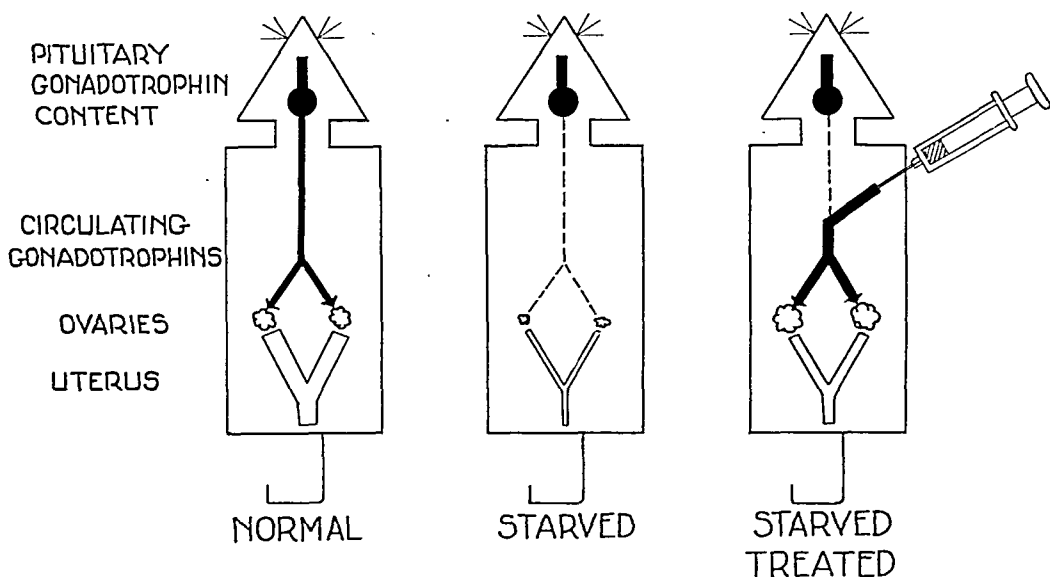
FIG. 1.

anterior pituitary twice a day for 3 days (from the 9th through the 11th day of starvation). A second series of rats (8 months old) were starved for 16 days and compared with fed controls.

Pituitary gonadotrophin content was measured by suspending each anterior pituitary gland in 6.0 cc of water by repeatedly drawing into and expelling from a syringe, then injecting into one 22- to 25-day-old Sprague-Dawley female rat, 1.0 cc twice daily for 3 days. The assay rats were killed 24 hours after the last injection.

Gonadotrophic hormone for injection into the starved adult rats was obtained from the anterior pituitary glands of adult rats castrated 10 months previously. The anterior pituitary glands were suspended in sufficient water so that injecting 0.5 cc twice daily for 3 days equalled administering one half gland.

Results are tabulated in Table I and represented schematically in Fig. 1.

Discussion. The starved rats reacted as if they had been hypophysectomized in that ovaries, uteri and vaginal epithelium underwent marked atrophy. They did not differ from hypophysectomized rats as concerns their potential capacity to respond to exogenously administered gonadotrophins, since administration of rat pituitary suspension,

rich in gonadotrophins, restored ovarian, uterine and vaginal cells to normal. Therefore, the conclusion that the circulating blood was low in gonadotrophin content seems warranted.

Pituitary content of gonadotrophin, measured directly, was not below normal. In fact, judged as potency per milligram of gland tissue, the starved rats' pituitary glands (since anterior pituitary weight had fallen circa 40% during starvation) were more potent than normal.

Thus a clear-cut dichotomy between pituitary content of gonadotrophin (normal amounts) and the amount of circulating gonadotrophins (low levels) has been established.

Pituitary content of gonadotrophin is the resultant of the relationship between the amount *produced* and the amount *released*. In acute starvation it is plain that failure of release occurs, as no peripheral gonadotrophin activity is detectable. Production of gonadotrophins must also be decreased since despite the lack of release, excesses are not accumulated within the gland. It is unlikely that the gonadotrophins would remain unchanged within the hypophysis for the entire experimental period of 12-16 days. It is more likely that the release mechanism fails completely and

otrophins appears to drop markedly during starvation as judged by ovarian atrophy, uterine atrophy, and anestrus vaginal smears in rodents,[1-7] and amenorrhea in women. The target organ atrophy is not due to a refractory state of the ovaries but to absence of gonadotrophins. This is suggested by the capacity of the ovaries of starved rodents to respond to injections of gonadotrophins[1,3,4,6,7] and the reduced urinary gonadotrophin output in women.[8,9]

Hypophyseal content of gonadotrophins has been determined in chronically starved rats and found to be decreased by Mason and Wolfe[2] and Werner[4] and not changed from normal by Marrian and Parkes.[1] In more acutely starved rats Pomerantz and Mulinos[10] could detect no decrease from normal. Each group of investigators used the method of implanting entire pituitary glands into the recipient assay rats. Since, following implantation, necrosis, growth and elaboration, or encapsulation may occur, the delivery of gonadotrophic hormone is exceedingly variable. This alone may account for the divergent results reported.

Methods. Adult (5 months old) Sprague-Dawley female rats were divided into three groups: Control rats fed *ad libitum;* rats starved for 12 days; and rats starved for 12 days and injected with a suspension of rat

1 Marrian, G. F., and Parkes, A. S., *Proc. Roy. Soc. London,* 1929, **105b,** 248.

2 Mason, K. E., and Wolfe, J. M., *Anat. Rec.,* 1930, **45,** 232.

3 Mulinos, M. G., Pomerantz, L., Smelser, J., and Kurzrok, R., Proc. Soc. Exp. Biol. and Med., 1939, **40,** 79.

4 Werner, S. C., Proc. Soc. Exp. Biol. and Med., 1939, **41,** 101.

5 Mulinos, M. G., and Pomerantz, L., *J. Nutrition,* 1940, **19,** 493.

6 Stephens, D. J., and Allen, W. M., *Endocrinology,* 1941, **28,** 580.

7 Drill, V. A., and Burrill, M. W., *Endocrinology,* 1944, **35,** 187.

8 Klinefelter, H. F., Jr., Albright, F., and Griswold, G. C., *J. Clin. Endocrinol.,* 1943, **3,** 529.

9 Jungck, E. C., Maddock, W. O., and Heller, C. G., *J. Clin. Endocrinol.,* 1947, **7,** 1.

10 Pomerantz, L., and Mulinos, M. G., *Am. J. Physiol.,* 1939, **126,** 601.

TABLE I.

Procedure	No. rats	Body weight			Donor rats				Recipient assay rats					
		Before g	After g	% change	Ant. Pituit. wt, mg	Uterine wt, mg	Ovarian wt, mg	Vaginal smears	Uterine wt, mg		Ovary wt, mg	No. rats		
									with fluid	without fluid				
Controls fed *ad lib.*	7	219	229	+5	0.7	501	63.0	cycling	175	110	21.3	7		
Starved 12 days	5	223	142	—36	6.1	195	30.3	atrophic in 4 days (2-6 days)	321	152	28.2	5		
RAP* days 9 to 11	6	222	137	—38	9.6	368	73.0	cornified following RAP inj.	302	145	20.2	6		
								Uninj. assay rats	41	41	15.6	9		
Controls fed *ad lib.*	5	257	261	+2	12.5	646	67.1	—	225	130	13.0	5		
Starved 16 days	7	253	156	—38	8.6	202	26.1		248	124	-14.5	7		
								Uninj. assay rats	42	42	13.5	10		

* Rat anterior pituitary gland suspensions, derived from rats castrated 10 months previously, were injected twice daily for 3 days, a total of ½ gland per rat.

subculture in media containing increasing quantities of this agent. A review of the literature on this subject is not included in this paper; for this, the reader is referred to the recent article of Spink and Ferris.[1]

The development of two kinds of resistance to penicillin in *Staph. aureus* has been demonstrated by Spink and Ferris.[1] The first type is an adaptation that can be reproduced *in vitro* by culturing in media containing increasing quantities of penicillin. The resistance is only temporary, can be abolished by growing the organisms in antibiotic-free broth, and cannot be associated with the production of penicillinase. The second, which occurs in patients as a result of treatment with penicillin, gives rise to strains of *Staph. aureus* in which the resistance is permanent and cannot be decreased by cultural manipulation; these organisms produce penicillinase.

Reports of penicillin resistance in *Strep. pyogenes* are few. One strain of this organism which appeared to be naturally resistant to penicillin (sensitive to 0.625 units per cc) was isolated by Hirsch *et al.*[2] from a patient with scarlet fever. Resistance was present when therapy with the antibiotic agent was first instituted and did not develop as a result of treatment. *Strep. pyogenes* was made resistant to penicillin *in vitro* by McKee and Houck.[3] They decreased the sensitivity of 3 strains of *Staph. aureus,* one strain each of types I, II, and III of *D. pneumoniae* and one strain of *Strep. pyogenes* to penicillin by growing the organisms in broth containing increasing amounts of this drug. The resistance of the beta hemolytic *Streptococcus* was increased 30-fold in a period of 3 months; there was an accompanying loss of virulence. Thirty-two rapid transfers in plain broth or 2 months' storage in the icebox produced no alteration in the degree of acquired resistance. Reduced velocity of growth and variation in colonial

form were observed during cultivation in media containing increasing amounts of antibiotic, but the organisms grew luxuriantly after transfer to fresh broth or to blood agar plates which contained no drug. No change in type of enzyme activity was apparent although fermentation reactions were much delayed.

The purpose of the present paper is to report the development of resistance in several strains of *Streptococcus pyogenes* following rapid subculture in broth containing increasing amounts of penicillin and to demonstrate the temporary nature of this resistance as shown by its abolishment by rapid transfer in broth containing no drug. The mechanism of the development of penicillin resistance in bacteria is controversial and will not be discussed here. This subject is reviewed in detail in the papers of Luria,[4] Demerec,[5] and Spink and Ferris.[1]

Methods. Fifteen strains of *Streptococcus pyogenes* recently isolated from the pharynges of patients with scarlet fever were the organisms used in this study. All of the cultures were kept on blood-heart infusion-yeast-tryptose agar and transferred once a week, being kept in the icebox in the interim. Penicillin resistance was determined by the method of Rammelkamp.[6] Solutions of penicillin for incorporation into media were made up freshly each week and kept in the icebox when not in use. The medium in which penicillin resistance was produced consisted of double strength heart infusion-yeast-tryptose broth (0.5 cc) to which was added 0.5 cc of the desired dilution of penicillin and 0.1 cc of a 24-hour culture of *Streptococcus pyogenes.*

All the strains of *Streptococcus* were grown first for 24 hours in broth containing no drug and their resistance to penicillin determined, using an inoculum of between 10,000 and 100,000 organisms per cc. One-tenth cc of a 24-hour broth culture of each was then

1 Spink, W. W., and Ferris, V., *J. Clin. Invest.,* 1947, 26, 379.

2 Hirsch, H. L., Rotman-Karka, G., Dowling, H. F., and Sweet, L. K., *J. Am. Med. Assn.,* 1947, 133, 657.

3 McKee, C. M., and Houck, C. L., PROC. SOC. EXP. BIOL. AND MED., 1943, 53, 33.

4 Luria, S. E., PROC. SOC. EXP. BIOL. AND MED., 1946, 61, 46.

5 Demerec, M., *Proc. Nat. Acad. Sci.,* 1945, 31, 16.

6 Rammelkamp, C. H., PROC. SOC. EXP. BIOL. AND MED., 1942, 51, 95.

early in starvation and that the eventual content reflects minimal production.

The mechanism controlling production and release of gonadotrophic hormone during starvation has not been elucidated. The possibility that the amount of circulating gonadotrophins or estrogens are major factors in controlling pituitary content during starvation seems unlikely since gonadotrophin content remains approximately the same despite wide variations in the amount of circulating estrogens and gonadotrophins (high in gonadotrophin injected starved rats, low in starved rats and normal in normal rats).

As a general proposition, release, circulating amount and excretion of gonadotrophins tend to parallel each other. For example, following castration, pituitary content, amount in the circulation and amount in the urine are decidedly greater than for intact animals for all species tested. Following administration of large (unphysiological) amounts of steroid sex hormones to either castrates or normal animals, pituitary content, amount in the circulation and urinary excretion of gonadotrophins fall below normal. Investigation of the 13-lined ground squirrel has revealed that during the period of sexual inactivity (hibernation) gonadal atrophy is associated with decreased amounts of gonadotrophic hormones in the pituitary and in the circulating blood. Just preceding and during the period of sexual activity, there are increased amounts of gonadotrophins in the hypophysis and circulation.[11]

Exceptions to this general proposition, other than the dichotomy found in starvation, have been demonstrated. Lauson, Golden and Sevringhaus[12] observed that immature female albino rats just prior to the onset of puberty had high pituitary levels of gonadotrophins before more than negligible amounts appeared in the circulation. At puberty, release was affected, and with diminishing content, increasing circulating amounts were noted.

Summary. During starvation circulating gonadotrophins fall precipitously whereas the content of hypophyseal gonadotrophin remains as high as under normal circumstances. This is in direct contrast to the usual situation in which pituitary gonadotrophin content accurately reflects the amount released into the circulation.

[11] Moore, C. R., Simmons, G. F., Wells, L. J., Zalesky, M., and Nelson, W. O., *Anat. Rec.*, 1934, 60, 279.

[12] Lauson, H. D., Golden, J. B., and Sevringhaus, E. L., *Am. J. Physiol.*, 1939, 125, 396.

16170

In vitro Development of Temporary Penicillin Resistance in Streptococcus Pyogenes.

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The treatment of bacterial infections with penicillin rarely leads to an increase in the resistance of the causative organisms to the antibiotic agent during the course of therapy. While such a phenomenon has been described

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for *Staph. aureus* and *Strep. viridans*, there is no substantiated evidence that it occurs with *Strep. pyogenes*, *D. pneumoniae*, *N. gonorrheae* or other organisms *in vivo*. *In vitro* studies, however, have shown that bacteria may be made less sensitive to penicillin by certain cultural manipulations. Thus, strains of *Staph. aureus*, which were originally susceptible to relatively small doses of penicillin, have been made quite resistant by repeated

subculture in media containing increasing quantities of this agent. A review of the literature on this subject is not included in this paper; for this, the reader is referred to the recent article of Spink and Ferris.[1]

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2 Hirsch, H. L., Rotman-Kavka, G., Dowling, H. F., and Sweet, L. K., *J. Am. Med. Assn.,* 1947, **133**, 657.

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5 Demerec, M., *Proc. Nat. Acad. Sci.,* 1945, **31**, 16.

6 Rammelkamp, C. H., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 95.

inoculated into a tube of broth to which had been added the lowest concentration of penicillin which was inhibitory, as well as into 2 other tubes, one of which contained 20% and the other 40% more drug than the lowest dilution in which the strain grew. The cultures were incubated at 37°C for 24 hours and 0.1 cc of the one showing growth with the largest amount of penicillin was subcultured to 2 other tubes, one of which contained the amount of penicillin which previously had just allowed growth to occur and the other, 120% of this quantity of the drug. This procedure of culturing in the highest concentration of penicillin allowing multiplication of the organisms and in 120% of this amount was repeated every 24 hours except for weekends, when the transfers were made every 48 hours. All cultures showing growth were plated on blood agar every 3 days in order to check the purity of the strains and to determine any changes in colonial or cellular morphology.

After 41 transfers in penicillin-containing broth, 6 strains, the 3 with the highest and the 3 with only moderate increase in resistance to penicillin, were subcultured 16 times more in an attempt to increase to a greater degree their ability to withstand the antibiotic agent.

Following demonstration of penicillin resistance in these 6 strains of *Streptococcus pyogenes* (after 57 transfers), the organisms were cultured in broth containing no drug. Transfers were made every 24 hours and susceptibility to penicillin determined after 10 and 37 subcultures.

Results. The results indicating the development of resistance to penicillin by the strains of *Streptococcus pyogenes* studied are presented in Table I. The data demonstrating loss of resistance to the antibiotic agent following frequent subculture in drug-free broth are shown in Table II.

Determination of penicillin resistance after 41 subcultures in blood-heart infusion broth containing increasing amounts of penicillin revealed 3 strains with no appreciable alteration in their susceptibility to the drug, although they were approximately 2 times more

resistant to penicillin than at the beginning of the experiment. This degree of change is within the limit of experimental error and cannot be considered significant. The resistance to penicillin of 4 strains (S-3, S-106, S-46 and S-47) increased 4-fold and of 5 strains (S-1, S-4, S-5, S-7, J-s) 8-fold after 41 transfers in penicillin-containing broth. One strain (S-9) was inhibited only by 16 times and 2 others (S-6, S-48), by 32 times the amount of antibiotic agent which stopped their growth at the start of the study.

Sixteen additional subcultures of 6 of the strains, a total of 57 transfers in drug-containing medium, revealed no further significant increase in penicillin resistance. One strain (S-47) revealed a 2-fold increase but this is within the limit of experimental error and, therefore, probably not significant.

Studies of the colonial and cellular morphology of the organisms at varying times during repeated transfer in penicillin-containing broth revealed no important changes. All of the strains produced matt colonies before and after being made drug-resistant. The colonies of the penicillin-insensitive variants were somewhat smaller than those of the drug-susceptible strains, although they varied considerably in size. Growth in broth and on blood agar plates was less rapid after frequent transfer in penicillin-containing media. No remarkable change in the ability of the *Streptococci* to produce hemolysin after developing resistance to the antibiotic agent was noted. The cellular morphology of the strains, as revealed by the Gram stain, underwent no alteration during the period of decrease in susceptibility to penicillin.

In order to determine whether or not the penicillin resistance which had been produced was temporary or permanent, 6 of the strains which had become relatively insensitive to drug were subjected to rapid subculture (every 24 hours) in broth containing no antibiotic agent. Determination of sensitivity to penicillin was carried out after 10 subcultures and revealed no significant change except with one strain which lost 50% of its resistance; this degree of change is, however, within the limits of experimental error. A

TABLE I.

Development of Resistance to Penicillin by *Streptococcus pyogenes* Following Rapid Transfer in Broth Containing Increasing Amounts of Penicillin.

Strain	Original resistance to penicillin, units/cc	Penicillin resistance after 41 transfers in broth containing penicillin, units/cc	No. of times more resistant to penicillin than original strain	Penicillin resistance after 57 transfers in broth containing penicillin, units/cc	No. of times more resistant to penicillin than original strain
S-1	.0075	.06	8x	—	—
3	.015	.06	4	—	—
4	.0075	.06	8	—	—
5	.015	.125	8	.125	8x
6	.015	.50	32	.50	32
7	.015	.125	8	.125	8
9	.015	.25	16	.25	16
10	.03	.06	2	—	—
106	.015	.06	4	—	—
J-s	.0075	.06	8	—	—
S-46	.015	.06	4	—	—
47	.015	.06	4	.125	8
48	.0075	.25	32	.25	32
49	.015	.03	2	—	—
50	.015	.03	2	—	—

TABLE II.

Loss of Penicillin Resistance of *Streptococcus pyogenes* After Frequent Subculture in Plain Broth.

Strain	Original resistance to penicillin, units/cc	Resistance to penicillin after 57 subcultures in broth containing penicillin, units/cc	Resistance to penicillin after 10 subcultures in plain broth, units/cc	Resistance to penicillin after 37 subcultures in plain broth, units/cc
S-5	.015	.125	.06	.015
6	.015	.50	.50	.015
7	.015	.125	.125	.0075
9	.015	.25	.125	.0075
47	.015	.125	.125	.015
48	.0075	.25	.125	.0075

striking increase in sensitivity to penicillin was present, however, after 37 subcultures in drug-free blood broth; all of the organisms returned to their original degree of susceptibility to the antibiotic. In the transformation from a drug-resistant to a susceptible state there was no change in the colonial or cellular morphology of the bacteria. The penicillin-sensitive streptococci appeared, however, to multiply somewhat more rapidly and to be more hemolytic than the resistant ones.

Summary and Discussion. The resistance of 12 out of 15 strains of *Streptococcus pyogenes* to penicillin was increased by daily subculture (41-57 times) in broth containing increasing amounts of penicillin, the maximum effect being obtained after 41 transfers; no significant increase was noted after 16 additional subcultures. The decrease in susceptibility to the antibiotic agent of 6 of the strains was temporary and was abolished rapidly by culture in drug-free media. No striking effects on the colonial and cellular morphology, degree of hemolysis, or rate of growth could be demonstrated during the process of acquisition or loss of sensitivity to penicillin. The drug-resistant strains, however, grew somewhat more slowly, produced slightly smaller colonies, and were a little less hemolytic than the highly susceptible

ones. No studies of penicillinase production were carried out; in view of the results of Spink and Ferris with *Staphylococcus aureus*, similar investigations on susceptible and *in vitro*-produced resistant strains of *Streptococcus pyogenes* would be very interesting.

Although one strain of *Streptococcus pyogenes*, which was relatively resistant to penicillin, has been described[2] this organism was isolated from a patient before therapy with the antibiotic agent was instituted. There have been no reports of the development of decreased susceptibility to penicillin during treatment of patients with streptococcal (Group A) infections with this drug. Hartman and Weinstein,[7] in a study of over 100 strains of *Streptococcus pyogenes* isolated from cases of scarlet fever were unable to

demonstrate any abnormal degree of resistance in the strains originally isolated or in those recovered from patients who had been treated with penicillin and in whom recurrence of *Streptococci* in the pharynx had occurred after therapy was stopped. It would appear from these results, therefore, that the development of resistance to penicillin by *Streptococcus pyogenes* may be easily accomplished *in vitro*, but occurs only rarely, if ever, in patients treated with this agent.

Conclusions. 1. The resistance of *Streptococcus pyogenes* to penicillin has been increased from 4 to 32 times by frequent subculture in media containing increasing amounts of the antibiotic agent.

2. The resistance to penicillin which develops *in vitro* is only temporary and can be abolished fairly rapidly by frequent subculture in drug-free media.

7 Hartman, T. L., and Weinstein, L., unpublished data.

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Temperature Regulation in Albino Rats Correlated with Determinations of Myelin Density in the Hypothalamus.[*]

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Newborn albino rats are unable to regulate their internal temperatures. When placed in a cold environment their temperatures descend rapidly to that of the environment; a warmer than normal environment results in a marked increase in internal temperature. It has been found that the ability of the young rat to regulate its internal temperature when placed in a cold environment gradually improves from birth to the age of 18-30 days, after which time it usually maintains its body temperature at or very near the normal level.[1]

It has been conclusively demonstrated that the hypothalamus is essential to temperature regulation.[2] Considerable numbers of small

myelinated fibers are consistently found in the lateral hypothalamus of the adult brain.[3] In spite of the diverse opinions which have appeared in the literature for many years regarding the correlation of function of neurons with myelination of their axons, it has been considered worthwhile to investigate myelination in the hypothalamus and to attempt correlation of the development of myelin with the beginning of the temperature regulating function of this portion of the brain.

The early literature concerning myelination

1 Hill, R. M., *Am. J. Physiol.*, 1947, **149**, 650.

2 Ranson, S. W., *Res. Publ. Assn. Nerv. Ment. Dis.*, 1940, **20**, 342.

3 Ingram, W. R., *Res. Publ. Assn. Nerv. Ment. Dis.*, 1940, **20**, 195.

of nerve fibers and possible correlations between myelination and function has been reviewed by Tilney and Casamajor[4] who also studied somatic activity in the kitten and correlated their observations with the distribution of myelin sheaths in the different fiber paths. They concluded from their investigation that myelinogeny in connection with behavioral components affords strong evidence in the cat that the deposition of myelin is coincidental with the establishment of function in definite fiber systems. They conceded that this coincidence might not, however, be the case in white rats in which both Watson[5] and Donaldson[6] reported complete absence of myelin in the nervous system at birth.

Langworthy[7] studied decerebrate preparations of kittens, young rabbits, and young guinea pigs. He found that myelination in the lower portion of the brain stem and the spinal cord was incomplete in kittens and rabbits in which decerebration was followed by movements of prolonged progression. In more mature animals in which decerebration was followed by the development of typical extensor rigidity, myelination of the long tracts was far more complete. He considered that correlation between myelination of the rubrospinal tract and the occurrence of decerebrate rigidity was strongly indicated.

Langworthy[8] also studied the behavior of pouch-young opossums and attempted to correlate their behavior with myelination of tracts in the central nervous system. He wrote, "The evidence that tracts in the central nervous systems become medullated at the time when they become functional is certainly suggestive, but by no means positive. No one who studies the opossum can evade the fact that this animal is capable of complicated reflex activity before any myelinated fibers are present." Angulo,[9] on the basis of observations of discrete reflexes in rat fetuses, stated that myelination is not a criterion of "functional insulation." Windle, Fish, and O'Donnell,[10] studying cat fetuses of ages from 42 to 60 days after insemination, found a high degree of reflex activity to be present in the fetuses even though the tracts involved were still unmyelinated. They concluded that myelination is not correlated with function in the absolute sense, but conceded that conduction in a given pathway may be improved after myelin sheaths have been acquired.

Ulett, Dow, and Larsell[11] investigated the inception of conductivity in the corpus callosum and the cortico-ponto-cerebellar pathway in young rabbits. They found that myelination of the fiber systems studied is not essential for the conduction of nerve impulses produced by electrical stimulation, and that conduction of such impulses precedes myelination of the fibers by several days in the rabbit. They did, however, observe an increased capacity for conduction in the larger and older animals.

Methods and Materials. Records of the temperatures of rats subjected to an environmental temperature of 5° to 8°C have been obtained through the use of iron constantan and copper constantan thermocouples sealed within the tips of ureteral catheters. Such catheters are easily inserted through the anus and rectum into the colon of rats as young as 4 days of age. The e.m.f. developed by the thermocouple at varying internal temperatures of the animal was originally measured with a Leeds-Northrup potentiometer. Each thermocouple was carefully calibrated with reference to the potentiometer before being used for the recording of temperatures. More recently temperatures have been directly recorded by means of a Brown electronic recording potentiometer. All temperature determinations were made with the

4 Tilney, F., and Casamajor, L., *Arch. Neur. and Psychiat.*, 1924, **12**, 1.

5 Watson, J. B., *Animal Education*, Chicago Univ. Contrib. to Phil., 1903, No. 4.

6 Donaldson, H. H., *American Textbook of Physiol.*, Phila., 1901.

7 Langworthy, O. R., *Carnegie Inst. of Washington, Contrib. to Embryol.*, 1926, No. 89, **17**, 125.

8 Langworthy, O. R., *J. Comp. Neur.*, 1928, **46**, 201.

9 Angulo, A. W., *J. Comp. Neur.*, 1929, **48**, 459.

10 Windle, W. F., Fish, M. W., and O'Donnell, J. E., *J. Comp. Neur.*, 1934, **59**, 139.

11 Ulett, G., Dow, R. S., and Larsell, O., *J. Comp. Neur.*, 1944, **80**, 1.
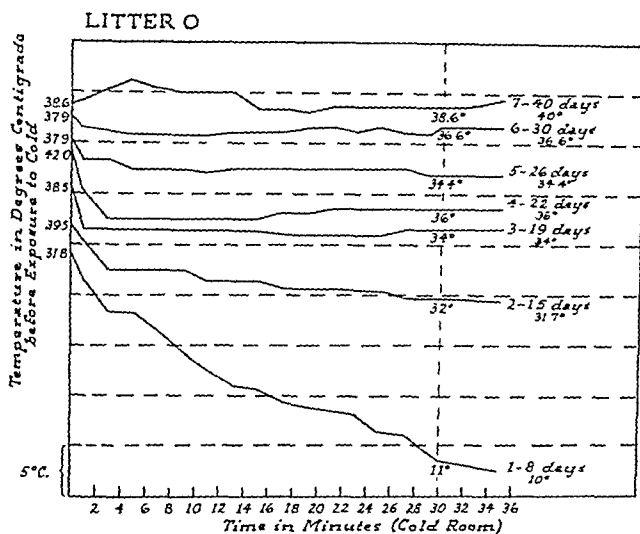
FIG. 1.
Temperature curves obtained from the members of one litter (Litter O) during 35 minutes in the cold room (5°-8°C). The ages (in days) of the respective animals are indicated.
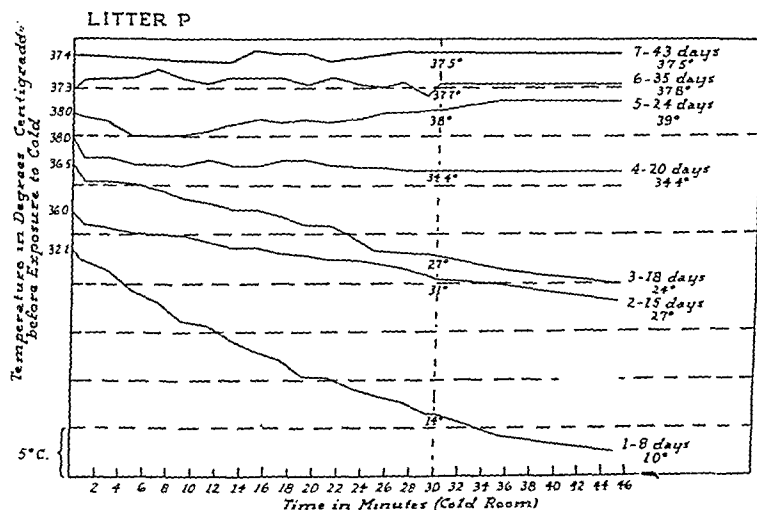
FIG. 2.
Temperature curves obtained from the members of Litter P during 45 minutes of exposure to cold (5°-8°C).

thermocouple inserted to the level of the diaphragm, thus assuring high colonic rather than rectal temperatures. It has been shown that such temperatures more accurately represent the thermal condition of the animal than those obtained by rectum, since the latter fluctuate with changes in the environmental temperature.[1]

The experimental procedure employed in our attempts to correlate temperature regulation with myelination of the hypothalamus has been carried out on twenty litters of Wistar strain rats. When a given litter had reached the age of 6 to 8 days, one animal from the litter was subjected to a cold environment (5-8°C) after first determining

its body temperature in a room whose temperature was approximately 26.5°C. The temperature of the animal was recorded at two-minute intervals with the Leeds-Northrup potentiometer, or at one-minute intervals with the Brown electronic potentiometer. Temperatures were recorded during 30 to 45 minutes. In the preparation of Fig. 3, 4, and 5, the temperatures after 30 minutes in the cold room were used to illustrate graphically the difference between normal environmental temperatures and those resulting from exposure to cold. Following exposure to cold, the animal was anesthetized with ether and perfused with one of three fixatives—10% neutral formalin, Erlicki's solution (copper sulphate and potassium bichromate), or 3½% potassium bichromate. The first of these preceded the Weil stain for myelin,[12] the second is the fixative recommended by Kultschitzky for his myelin stain,[13] and the third is that found most satisfactory for the myelin stain used by Ulett and his collaborators.[11] After perfusion, the brain was removed, fixation was continued for the specified period, and in the solutions specified by the originator of the stain. A block from the center of the brain which included the hypothalamus, or a complete half brain was finally imbedded in paraffin, sectioned, and mounted on slides. Other animals from the same litter were similarly treated at appropriate intervals until the litter was exhausted. The same fixatives were applied in a uniform manner to the brains of all the members of a given litter. When all the hypothalami from a given litter had been sectioned at as uniform a thickness as is possible with the Spencer rotary microtome and placed on slides, staining of the sections was carried out. In order that comparisons based on the density of the myelin stain could be made between the hypothalami of the rats of different ages, three slides (9-12 sections) were selected from each animal in the litter. These were placed in a staining rack and stained en masse. Theoretically, if
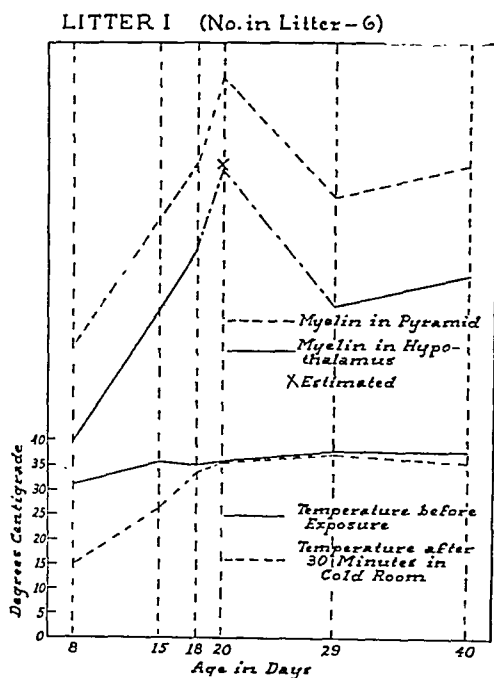
FIG. 3.

Records of temperatures before and after 30-minute exposures to cold and curves representing the density of myelin staining in the pyramid and lateral hypothalamus as determined photometrically. (Technical difficulties, resulting in loss of sections of the hypothalamus in the 20-day animal, made an accurate determination impossible.)

the stain is specific for myelin, those hypothalami containing the least myelin (or no myelin) should be less deeply stained than those in which myelination is further advanced. In our experience the myelin stain reported by Ulett, Dow and Larsell[11] has given the most satisfactory results, since the nerve cells remain almost entirely unstained in sections prepared according to their method.

Determinations of staining density have utilized a photometric method. The light from a Spencer microscope lamp has been directed through a monochromator to the substage of a binocular microscope; the monochromator was set to deliver light at a wavelength of 600 mu. The phototube of a Photovolt electronic photometer is firmly attached to the right ocular of the microscope in such a manner that all light traversing that ocular

12 Weil, A., *Arch. Neur. and Psychiat.*, 1928, 20, 392.

13 Lee, B., *The Microtomist's Vade-Mecum*, 10th ed., p. 525.
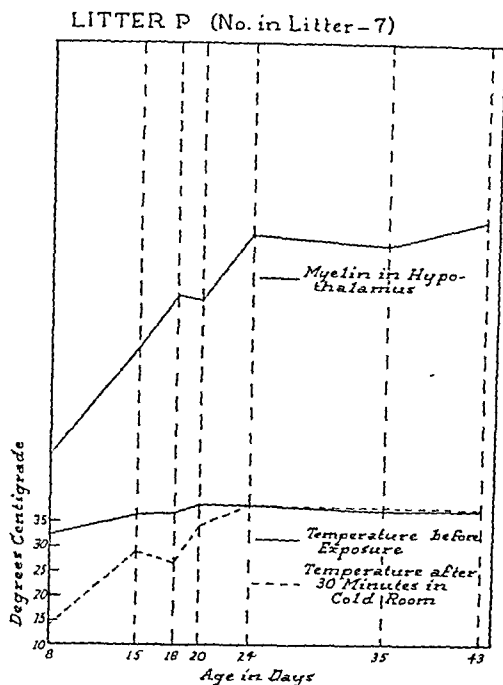
FIG. 4.

Records of temperatures before and after 30-minute exposures to cold and curve representing the density of myelin staining in the lateral hypothalamus in Litter P. (*Cf.* Fig. 2.)

FIG. 5.

Records of temperatures before and after 30-minute exposures to cold and curves representing the density of myelin staining in the pyramid and lateral hypothalamus in Litter O. (*Cf.* Fig. 1.)

is focused upon the photoelectric cell and thus recorded directly upon the galvanometer of the instrument. The procedure for obtaining the density of the myelin stain in a given section consisted of focusing upon the lateral hypothalamic area through the left ocular of the microscope after which the slide was moved to an area free of sections; without altering the focal adjustment of the microscope, the left ocular was covered and the light entering the microscope was adjusted by means of a rheostat and the iris diaphragm at the inlet of the monochromator to give an arbitrary galvanometer reading, which "zero" reading was then kept constant for an entire series of determinations. The maintenance of a constant "zero" point on the scale compensated for differences in thickness of slides, cover slips, and mounting media. After the light had been adjusted, the section was moved back into the field, the left ocular was again covered, and the galvanometer reading recorded. The difference between this reading and the "zero" reading repre-

sented the amount of light absorbed by the stained section. This procedure was repeated on several sections of the hypothalamus of a given animal, and the several readings were then averaged. The averages were used in the construction of myelin curves for the litter being studied. Typical myelin curves obtained by this method in three of the litters investigated are shown in Fig. 3, 4, and 5. Myelin determinations have also been made on the pyramid of the medulla in several of the litters studied (Fig. 3 and 5) in order to check myelination in an area completely free of nerve cell bodies.

Results. The temperature curves during exposure to cold in animals from the same litter at progressively older ages are illustrated in Fig. 1 and 2. These are entirely consistent with previous obervations by one of us[1] and show a progressive improvement in ability to regulate against cold. Regulation, aside from an initial drop of some 3½ degrees, was fairly well established in animal 3 (Fig. 1) at 19 days of age, and was completely established

in animals 6 and 7 at 30 and 40 days of age. Regulation in Litter P (Fig. 2) was quite good in animal 4 at 20 days of age, and was completely established in animal 5 at 24 days of age.

In the majority of litters studied, very adequate regulation against cold was evident at 18 and 20 days of age (Fig. 3). In some litters complete regulation appeared later (24 days in Litter P. Fig. 4, and at 30 days, Litter O, Fig. 5). The density of myelin stains as measured by the method described above has regularly reached a high level coincident with the beginning of regulation (Fig. 3, 4, and 5). In most of the litters studied, there has been a tendency for a levelling off or a depression of the myelin curve during the post-regulatory phase. As was to be expected, the pyramids of the medulla showed considerably greater degrees of staining density than the hypothalamus at all ages. There was, however, a striking parallelism between the curves representing density of the pyramids and of the hypothalamus in progressively older rats (Fig. 3 and 5).

The marked pre-regulatory depressions in the myelin curves for hypothalamus and pyramid in Litter O (Fig. 5) may have been due to a dietary deficiency, since we were unable to obtain lettuce for our rat colony at the time when this particular litter was born. The same dietary deficiency may have been responsible for the delay in attainment of regulatory ability and the corresponding delay in myelin formation as indicated by the fact that the curves reached their highest level in the 30-day animal.

Discussion. Koch and Koch[14] studied the chemical differentiation of the brain of the albino rat during growth. Their analyses were carried out on whole brains of rats 1, 10, 20, 40, and 120 days of age. The results of their investigation so far as phosphatides are concerned may be summarized as follows:

Age of rats in days	1	10	20	40	120
Phosphatides	15.2%	12.3%	21.4%	21.8%	21.6%

The phosphatides have been selected from their table as being the most likely substances having affinity for the various modifications of the Weigert stain for myelin which have been used in our experiments. The rather marked increase in phosphatides during the first 20 days compares favorably with the curves which have been obtained by our method of determining the concentration of myelin in the hypothalamus and pyramid of the albino rat. None of our investigations of myelin have been carried out on rats younger than 6 days of age, and we therefore have no material available for comparison with the figure of 15.2% phosphatides found in rats one day of age. It will be noted that Koch and Koch actually found a lower percentage of phosphatides in the brains of 10-day rats than in those one day old. The tendency for our myelin curves to flatten out or to be depressed during the period between the 20th and 40th days of life may possibly be due to the failure of phosphatides to increase appreciably during that period. Further studies are in progress to determine, if possible, the individual specificities of our myelin stains for lecithin and cephalin. Cholesterol will also be studied in this connection. Langworthy[15] called attention to the well known fact that animals, particularly those born in large litters, vary greatly in their maturity at birth and in the rapidity of their growth thereafter. We have studied litters of albino rats containing as many as 12 individuals, and have found that the attainment of the ability to regulate internal temperature when exposed to a cold environment is somewhat delayed in such large litters and that there is a tendency for the myelin curves to be much less steep in the period between 6 and 20 days than is the case in the smaller litters.

Watson[5] stated that myelination does not begin in the higher centers of the brain until rats have attained the age of 24 days. Although this may be true of cortical association fibers, it certainly does not appear to be true of the hypothalamus, if our method is valid for the determination of the presence of myelin.

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14 Koch, W., and Koch, M. L., *J. Biol. Chem.*, 1913, 15, 423.

15 Langworthy, O. R., *Carnegie Inst. of Washington, Contrib. to Embryol.*, 1929, No. 114, 20, 127.

The somewhat earlier development of resistance to cold in these animals (Wistar strain) as compared to those previously studied (Denver University strain) may be due to the difference in strain, or it may be due to the fact that in the earlier experiments lettuce was not a part of the diet of the mothers whereas it was in these experiments in all cases except Litter O (Fig. 5).

Conclusions. The optical densities of sections of the hypothalamus stained by modifications of the Weigert method have been measured in rats varying in age from 6 to 70 days by means of monochromatic light and an electronic photometer. These measurements have been correlated with the abilities of the animals to regulate their internal temperatures when exposed to a cold environment. We believe that our method has recorded densities which are dependent mainly upon the amount and/or character of the myelin in the hypothalamus at the respective ages studied, and that the increase in myelin from birth to 20 days of age may contribute to the acquisition of the capacity for temperature regulation. We do not believe that myelination in the hypothalamus is the only factor concerned in the acquisition of this function. Many other factors are no doubt operative and many of these are currently being investigated.

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Teratogenetic and Lethal Effects of Influenza-A and Mumps Viruses on Early Chick Embryos.

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From the Industrial Hygiene Research Laboratory and the Division of Infectious Diseases, National Institute of Health, Bethesda, Md.,[‡] and the Department of Zoology, Washington University, St. Louis, Mo.

Gregg[1] demonstrated a high incidence of congenital malformations in children whose mothers had contracted rubella (German measles) during the early months of pregnancy. These observations were confirmed by Swan[2,3,4] in Australia, and by American and European workers. Over 400 cases are now on record. The most common abnormalities caused by rubella are: cataract in one or both eyes, heart defects, microcephaly and deaf-mutism. The incidence of malformations following infection of the mother within the first 2 months of pregnancy is almost 100%, but low after the 4th month. It seems that a very early infection (around 1-1½ months of pregnancy) causes predominantly cataract, whereas a later infection (around the 2nd month) causes predominantly deaf-mutism.

The discovery that the rubella virus passes through the placenta and causes a specific pattern of localized malformations in the human embryo is of great interest from the point of view of embryology, virus biology and clinical obstetrics. It seemed to be of importance to analyze the effects of viruses as teratogenetic agents under the controllable conditions of animal experiments.

The chick embryo suggested itself as a suitable embryonic material, since it is known

1 Gregg, N. McA., *Trans. Ophthalmological Soc. of Australia*, 1941, 3, 35.

2 Swan, C., Tostevin, A. L., Moore, B., Mayo, H., and Barham Black, G. H., *Med. J. Australia*, 1943, 2, 201.

3 Swan, C., Tostevin, A. L., Mayo, H., and Barham Black, G. H., *Med. J. Australia*, 1944, 1, 409.

4 Swan, C., and Tostevin, A. L., *Med. J. Australia*, 1946, 1, 645.

that the tissues and the extra-embryonic membranes of the chick embryo are a favorable medium for the culturing of a number of viruses (Beveridge and Burnet[5]). Furthermore, the absence of a placenta in the chick makes a direct infection of the embryo possible. thus avoiding the complications introduced by an extra-embryonic barrier.

The rubella virus was not available. Instead. we used influenza-A virus (strain PR8) and mumps virus. Experiments with rubeola virus are in progress.

Developmental stages used. The routine methods of culturing viruses in eggs make use of chick embryos incubated for 8 or more days. In these stages the chief reservoirs for virus, namely, the allantoic and amniotic sacs are well developed. However, embryos of that age would be unsuitable for our purpose since most of their organs are far advanced in their differentiation. Teratogenetic effects can be expected only if the embryos are infected at much younger stages, that is, in the first phases of morphogenesis or in the still earlier stages of organ determination.

With the exception of a group of experiments on 4-day embryos all embryos used were incubated for approximately 48 hours in a forced-draft incubator at a temperature of 98°F. The youngest embryos had about 16 somites: their eyes were in the optic vesicle stage; the axis was straight and the heartbeat had just begun (Arey;[6] Fig. 508B). The oldest embryos had about 27 somites: their eyes were in the optic cup stage with lenses: 3 pairs of visceral arches were developed: the cranial and cervical flexures of the head and the torsion of the main axis were in progress; the amnion had overgrown the head approximately to the heart level (Arey:[6] Fig. 523B). Most embryos were in stages intermediate between these 2 (Arey;[6] Figs. 506C and 523B). All eggs came from

a Government controlled flock of New Hampshire Reds.

Technique of infecting 48-hour embryos. The egg was candled. and the position of the blastoderm was marked on the shell (the embryo is not yet recognizable at that stage). The egg was then placed on a Syracuse dish with a cotton-cushion. A square window, about ¼ inch in length and width was sawed in the shell over the blastoderm, using a hacksaw blade. The window was removed and the underlying shell membrane was ruptured after it had been thoroughly moistened. The embryo was thus exposed. Next, the transparent vitelline membrane which surrounds the entire yolk was ruptured over the embryo with a fine glass needle or a pair of watchmaker forceps. (Instruments described in Hamburger[8]). This membrane had been found to be a barrier to virus infections. Meanwhile, a tuberculin syringe (½ cc) with a 27-gauge needle had been filled with the standard dosage of 3/100 cc of the solution to be injected. The point of the needle was inserted through the hole in the vitelline membrane. and the liquid spread slowly over the embryo. Care was taken to hold the needle point close to the embryo but not to touch it nor to inject abruptly in order to avoid injuries to the embryo or blastoderm. The window was then closed by sealing with paraffin the piece of shell which had been previously removed. Incubation was continued in a Buffalo incubator without forced circulation, running at 99°-100°F. All instruments. glassware, and fluids were kept sterile. All eggs were candled each day.

Experiments with Influenza-A virus. Source of virus material. An egg-adapted PR8 strain of influenza-A virus was used. Each sample used had been tested for virus titer by Salk's modification of Hirst's red cell agglutination test. cultured for sterility and stored in flame-sealed ampoules in a frozen state at −73°C. The 4th to 7th egg-passages (p4 to p7) were used in our experiments. They had titered as

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[5] Beveridge. W. I. B., and Burnet, F. M., *Medical Research Council*, 1946, Special Report Series No. 256.

[6] Arey, L. B., *Developmental Anatomy*, Fifth edition. 1946, W. B. Saunders. Philadelphia, Pa., and London.

[7] Habel, Karl, *Public Health Rep.*, 1945, 60, 201.

[8] Hamburger. Viktor, *A Manual of Experimental Embryology.* 1942. The University of Chicago Press.

TABLE I.

Infection of 2-day Chick Embryos with Influenza-A Virus.

Exp.	Passage	Concn.	Total infected	Discarded	Revised total	Abnormal	Normal	Died, days after infection
5f1	p1 *	undil.	20	—	20	20	—	1 -2½
6f1	p1	1:4	23	5	18	18	—	1½-2
8f1	p5 †	1:10	25	1	24	11	13	1½-2
10f1	p5	1:100	25	1	24	3	21	12 were alive at 18 days
14f1	p5	1:4	28	3	25	25	—	1½-2½
15f1	p6	1:10	22	4	18	18	—	1 -3
16f1	p6	undil.	9	—	9	8	1	½
26f1	p6	1:4	10	1	9	7	2	2 -2½
27f1	p6	1:20	10	2	8	7	1	1½-2½
28f1	p6	1:50	9	—	9	9	—	2½-3
30f1	p7	1:4	48	1	47	47	—	1 -3
34f1	p7	1:100	12	—	12	12	—	½-1
35f1	p7	10-3	12	—	12	11	1	2 -3
36f1	p7	10-1	12	1	11	11	—	3 -3
37f1	p7	10-5	12	—	12	12	—	3 -3
38f1	p7	10-6	12	1	12	12	—	3 -3
39f1	p7	10-7	12	—	12	9	9	6 were alive at 5½ days
47f1	p7	1:100	10	1	11	9	1	2 -3
48f1	p7	1:100	8	—	10	8	—	1½-3
51f1	p7	1:100	20	—	8	20	—	2 -3
52f1	p7	1:10	20	1	19	19	—	1 -2½
Total			359	21	338	289	49	½-3

* Bracket indicates that the same sample was used.

† Sample had probably low virulence; see text.

follows (before freezing): p4 at 1:16000; p5 at 1:2048; p6 at 1:32000; p7 at 1:8000.

Experimental results. Virus-infected allantoic fluid was used in concentrations from un-

FIG. 3.
Case 15fl-4. Same as Fig. 2. Fixed at 3½ days of incubation. Stained with Delafield's Hematoxylin.
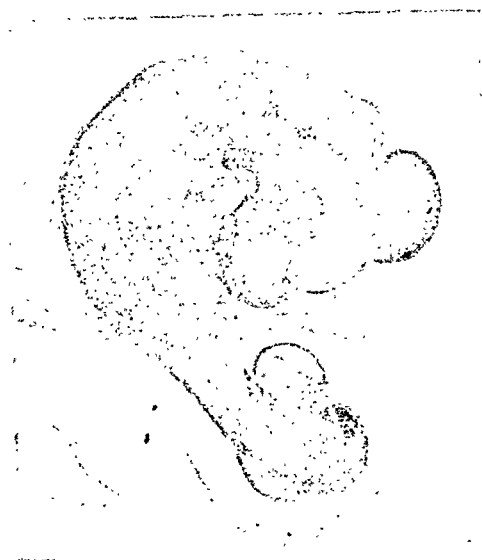
FIG. 1.
Normal 4-day chick embryo.
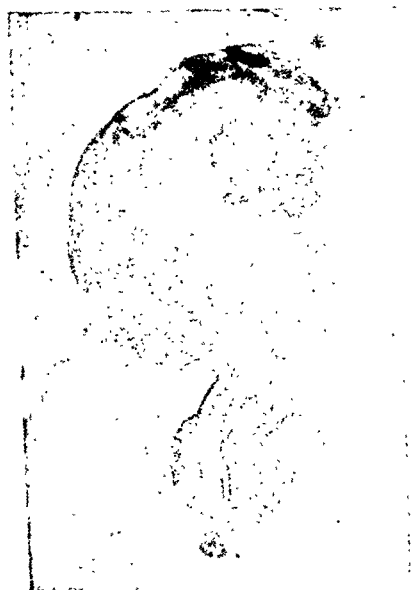
FIG. 2.
Case 15fl S. Infected with influenza-A virus. (PR8, p6, allantoic fluid diluted 1:10) at 2 days of incubation. Fixed at 4 days of incubation. Stained with Delafield's Hematoxylin.

diluted to 10^{-7}, as indicated in Table I. With the exception of 2 series, practically all embryos infected with dilutions up to 10^{-6} died within 3 days after infection (5 days of incubation), and showed severe abnormalities. The 2 exceptional series (8fl and 10fl) had been infected with the same sample of p5 diluted to 1:10 and 1:100 respectively. As was mentioned above, p5 had the lowest hemagglutination titer of the passages used. Since all other passages gave almost 100% lethal and teratogenetic effects at the same dilutions, it is assumed that this particular sample had an exceptionally low virulence. The predominance of normal embryos in 39fl indicated that the threshold of infectiousness is reached at a dilution of 10^{-7}. If all experiments with the exclusion of 8fl, 10fl, and 39fl are taken together, the incidence of lethal and teratogenetic effects amounts to almost 98% (273 of 279 cases). The 6 normal specimens have probably escaped an infection due to a faulty technique.

Description of abnormalities. All infected embryos were severely malformed. The following symptoms were observed macroscopically (see Figs. 2, 3). A histological exam-

ination of the material is in progress.

(1) The head and, in particular, the brain, are disproportionally small (*microcephaly, micrencephaly*). Tel-, di-, and mesencephalon are abnormal in shape. This is particularly striking in the case of the mesencephalon which is a large, protruding thin-walled vesicle in normal 3- to 5-day embryos, whereas in all infected embryos it is a flattened and unexpanded structure, showing a ring of whitish tissue at its base. The eyes seem to be less affected. They are pigmented in the older embryos and approach normal size which makes them appear disproportionally large. No striking gross abnormalities were found at the trunk and tail levels and in the limb buds.

(2) The main axis is more or less twisted in all infected embryos. In normal development, conspicuous flexures occur during the stages under consideration. First, a torsion of the head takes place in such a way that the left side faces the yolk. Simultaneously, the head undergoes a bending in the midbrain level (cephalic flexure) which brings the forebrain in a position almost parallel to the hindbrain. Shortly afterwards, a bending in the hindbrain level (cervical flexure) takes place. An additional temporary flexure occurs in the wing level which continues into the trunk level. As a result, an embryo of about 27 somites has the appearance of a question mark (?). By the 4th day, the flexures have been smoothed out and the dorsal axis forms a smooth circular curve (Fig. 1). In the infected embryos, the cervical flexure and the one in the wing level are more or less strongly accentuated. In the most severe cases, the main axis is folded upon itself, forming a tight sigmoid curve. There may be one or 2 twists in the same embryo. The points of excessive twisting vary from case to case, but they are always in the cervical, wing, or middle trunk levels.

(3) The amnion which grows out as a protective membrane during the stages following infection is severely impaired in its growth. The head fold of the amnion usually proceeds to cover the head, but the lateral and tail folds either fail to grow out altogether

or they do so at a greatly reduced rate. As a result the amnion rarely closes over the embryo, and gaps were found ranging from a small hole over the posterior end to the complete exposure of the embryo from the heart level to the tail end. In the few cases in which the amnion does close, it is very tight, the amniotic fluid is under high pressure, and, as a result, the lateral body walls which are continuous with the amnion are folded dorsad instead of ventrad, exposing the viscera, such as mesonephros and intestine (*ectopia viscerum*). It is possible that the severe twists of the main axis are wholly or in part, secondary effects of this failure of amniotic growth. The rapidly growing and stretching embryo seems to be crammed for space in the amniotic cavity; the underdeveloped amnion would offer a barrier, and the result would be a passive folding of the axial organs at the points where normally a slight bending occurs.

(4) The growth of the entire embryo seems to be impaired; however, this point requires verification by exact measurements.

Each symptom showed a moderate range of variability. However all symptoms appeared in all 273 affected specimens. The uniformity and reproducibility of the syndrome under the standard conditions of the experiment were striking. The circulatory system did not seem to be affected. The allantoic vesicle grew out normally in most instances.

The lethal effect seems to be an all-or-none effect in the sense that embryos which receive an infectious dose (dilutions to 10^{-6}) die within 3 days after infection, whereas embryos showing no abnormalities and surviving the 3rd day seem to have remained uninfected. Within this 3-day range, there seems to be a correlation between virus concentration and life span. In a dilution test, 6 sets of embryos (12 per set) were infected with serial 10-fold dilutions from 10^{-2} to 10^{-7} of the same sample. The embryos infected with a dilution of 10^{-2} died within one day after infection, those infected with 10^{-3} died within 1-2 days and those infected with 10^{-4} to 10^{-6} died within 2-3 days. The cause of

TABLE II. Control Experiments.

Type	Exp.	Conc.	Total	Discarded	Revised total	Died, days after 2-day stage		Alive, when opened, days after 2-day stage			Abnormal†	
						1-5	5½-12	1-5	5½-12	more than 12	Total	%
Untreated	49e	—	72	5*	67	3	1	2		63	3	4.5
	32e	—	54	1	53	9	13	16	1	28	2	5.8
0.9 saline	5efl	undil.	19	—	18	3		1				
	8efl	1:10	14	1	14	3	2	4	1	7		
Allantoic fluid	12efl	undil.	16	4	15	5		2		6	1	
	15efl	"	15	3	11	7				2	4	
	17e	"	24		21	9	5	3	1	6	1	
	18e	"	24		24	9	3		8	6	2	
	19e	1:4	7	1	7	3				3		
	20e	undil.	15	1	14	4	2		6		1	
	21e	"	15	3	14	4	4		5		2	
	22e	undil.	24		21	14	6					
Total: allantoic fluid			173	14	159	60	18	26	22	33	11	6.9
Ultraviolet irrad. virus	25e	undil.	32	4	28	11	5	2		9	2	7.1

* Sterile.
† All dead at 3-6 days of incubation.

death is obscure. As was mentioned, the circulation seems to be intact; there is evidence of a generalized infection.

Control Experiments (Table II). In order to test the incidence and types of abnormalities occurring in the egg material used for our experiments, 72 unopened eggs were incubated. Of these 5 were sterile, 3 were found dead and abnormal at 3½ days, one died at 12½ days and the remaining 63 embryos were opened and found to be normal on the 15th day of incubation. Of the abnormal embryos, one showed a twist in the cervical region and a slight microcephaly. However, the presence of a fully expanded midbrain, and other details, distinguish this embryo distinctly from the influenza-infected embryos. Another specimen had head abnormalities similar to the first one but no twist. In the third, the limb buds were bent upward, and the posterior trunk axis bent ventrad.

Injections of 0.9% salt solution resulted in an increase of the mortality during the early stages of development. The percentage of abnormalities was not higher than in untreated embryos. The 2 abnormal embryos of this series had died between 2 and 3½ days after treatment. They both had twists of the axis, and head abnormalities similar to those found in the untreated controls but different from those described for influenza-infected embryos.

Injections of uninfected allantoic fluid from normal 15-day embryos resulted in a slight increase in the incidence of abnormalities and in a marked increase of the mortality in early stages. Of the 11 abnormal embryos, 8 showed slight to severe twists of the axis. Malformations of the head were found twice in combination with twists, and twice in otherwise normal embryos. They were, again, of a type similar to those occurring in other controls but different from those found in virus-infected embryos. Furthermore, not a single case in any of the control series presented the combination of head, axis, and amnion deficiencies which is characteristic of all influenza-infected embryos. Hence, the symptoms described above must be considered as specific effects of the virus infections. The

fact that some of the abnormal control embryos exhibit a twist of the axis similar to infected embryos is readily understood; the virus infection interferes with the normal developmental processes at certain points of least resistance which are equally vulnerable to other teratogenic agents.

Evidence for virus multiplication in infected embryos. The following experiment shows that living virus and not its toxic products are instrumental in bringing about the teratogenetic effects. Samples of allantoic fluid (p5 and p6) virus were inactivated by ultraviolet light. The irradiated fluid was used undiluted for the injection of 28 embryos. Two abnormal embryos were found at 4 days of incubation (7%); both showed twists, and the one, in addition, a severe deformity of the hind end. The percentage of abnormalities and the mortality rate at early stages up to 12 days, were almost identical with those of the normal allantoic-fluid controls. The experiment shows that the characteristic effects of influenza-A virus are checked, if the virus is killed. Evidence that virus growth had taken place following infection was shown with 10 specimens of Exp. 30fl (Table I) collected when alive 2 days after infection and showing severe symptoms. They were removed from the blastoderm, pooled, ground in the Waring Blender and the supernatant titrated from 10^{-1} to 10^{-7} in 8-day embryos. The allantoic fluids harvested from the survivors after 6 days' incubation tested for hemagglutination indicated a titer of at least 10^{-7}. The actual titers were probably higher since one cc of saline per embryo was added before the dilutions were made. In addition a hemagglutination titer was made with the original embryonic tissue and was found to be 1:1024. Previous experiments have shown that the egg-passage strain of Influenza-A virus dies off rapidly in the liquid state when kept at room temperature. It is concluded that the virus present in the infected 4-day embryos is not merely due to survival of injected virus particles but that it is the result of multiplication.

Infection by transplantation of infected tissues. As was shown, the head, and, in particular, the brain are severely malformed, whereas the trunk and tail rarely show macroscopic defects. Since care was taken to obtain an equal spread of the infectious fluid, this differential effect seems to be due to inherent differences in tissue susceptibility. An attempt was made to alter the pattern of abnormalities, by means of the method of embryonic transplantation which allows one to bring infected tissues in close contact with any part of the embryo. The following technique was employed: Highly abnormal embryos were obtained by injecting allantoic fluid (p7), diluted to 1:4 and 1:100 respectively. A number of embryos were recovered when alive, at 4 days of incubation. They were removed from the blastoderm, transferred to a Syracuse dish, and small pieces of the midbrain or other brain parts were cut out with an iridectomy knife. These fragments were then implanted in different levels of normal 2-day embryos, that is, near the brain, near trunk somites, or in the hind limb or tail region (for further details of technique see Hamburger[8]). The host embryos were incubated for another 2 days. All embryos (altogether 26 cases) showed the characteristic symptoms of influenza-A virus infection, including microcephaly, no matter where the transplants had been placed. Some transplants had fused with the host tissues, for instance with the left hind limb bud, or with somites; others were found floating freely near the embryo, still others were not recovered at all. Control experiments with normal brain tissue gave no abnormalities. These experiments give further evidence of the presence of living virus in the brain tissue of infected embryos. Since the host embryos failed to show focal infections at the posterior parts of the body, following implantation of infected tissue in these levels, we assume that the virus has spread from the transplants to all parts of the body and that a selective infection of the brain has taken place. It seems that the embryonic brain offers a preferential medium for influenza-virus growth. Further direct evidence for a higher rate of virus multiplication in the anterior parts of the embryo as compared

to the posterior parts was obtained by titrating the emulsified anterior and posterior halves of 18 infected embryos by the hemagglutination technique. The anterior halves titered to 1:6400 and the posterior halves to 1:1600.

Infection of 4-day embryos with Influenza-A virus. In a preliminary experiment (23fl) a dose of 3/100 cc per embryo of allantoic fluid (p6, diluted 1:4) was injected directly into the extra-embryonic coelom, through a hole in the somatopleure between the wing and leg buds, after a window was sawed over the embryo, the latter placed on the candle and the syringe needle inserted through the slit for about one-half its length and aimed at the embryo. Since both techniques gave identical results, the latter, more simple procedure was used henceforth. Of 84 injected embryos, 74 were found dead between one and 3½ days after infection; the others were opened when alive for the purpose of preservation. The majority of the embryos appeared to be without gross abnormalities but a few exhibited slight twists of the axis, and a few others showed signs of brain infections.

Dilution tests were run parallel with those reported above for 2-day embryos, and again a correlation was found between concentration and life span. Even a dilution of 10^{-7} which was not fatal for 2-day embryos killed 7 of 8 four-day embryos within 3½ days after infection. It is clear from these experiments that 4-day embryos are at least as susceptible as 2-day and 8-day embryos. The absence of abnormalities in infected 4-day embryos is understandable since at the stage of injection the development of organs is advanced far beyond the 2-day stage, and the amnion is closed so that no twist of the main axis of the embryo should be expected if our explanation of the mechanics of twisting given above is correct.

Experiments with Mumps Virus. Source of the virus material. The material for one experiment (15mp) was derived from strain "M" (Habel[7]) which had been carried through 37 monkey-parotid passages and then established in the allantoic sac of the chick embryo. Injections for routine passages were made in 8-day embryos (2/10 cc per embryo) and

TABLE III.

Infection of 2-day Chick Embryos with Mumps Virus.

Exp.	Passage and strain	Conc.	Total	Died, days after infection							Preserved: alive 1-5 days after injection	Abnormalities		
				1	2	3	4	5	6	Total		Twists	Other	Total
12mp	4(EVI)	undil.	18		1	2		13		14	4	2		2
13	4	"	19	1		4		3		12	7	1	1	2
14	4	1:10	13		1	2	5	3		8	5		1	1
17	6	"	13	1	2	2	2	2	1	7	2		2	2
18	7	"	7		1	2	1	1		6	21	5	2	1
19	13	1:10	27	1	3	6	2	1	1	5	7			
	13	1:100	18	1	6	2				5	2			
15	17	undil.	7							11	1			7
38(M)			12	1		1	1	1	1	11		7		
		Total	134	7	16	21	11	20	1	85	49	13 = 11.2%	6	21 = 15.7%
Ultraviolet irrad. virus 16mp	13(EVI)	undil.	28		1	2	2	2		7	5 (16 alive 11 days after injection)		0	

the allantoic fluid harvested on the 18th day. The sample used in 15mp represents the 38th allantoic passage of "M." In all other experiments a more virulent strain was used which was originally derived from "M." It multiplied more rapidly in eggs and titered higher on hemagglutination and will be designated as "EVI." The samples of "EVI" used in our experiments came from allantoic sac passages 4, 6, 7, 13.

The technique of infection was the same as that used for influenza-A, the embryonic stages and the standard dose being the same.

Results. Of 161 infected embryos, 134 survived the operation (tabulated in Table III). Of these, 49 were preserved when still alive. The table shows that the mumps virus is lethal within 5 days after the infection of 2-day embryos. This effect is convincingly demonstrated by those experimental series in which all, or all but one or 2 specimens, had been allowed to continue development until death occurred. The 49 specimens which had been fixed during the first 5 days when still alive were unselected material. The percentage of abnormalities (15.7%) were higher than in allantoic fluid controls (6.9%). Twists of the axis were the most common malformations; they occurred with a particularly high incidence in Exp. 15mp in which the "M" strain was used. No case of microcephaly was observed. The wing and leg buds were abnormal in 4 cases, and a shortening of the beak was found in a few instances.

Evidence for virus multiplication in infected embryos. Infected allantoic fluid (16cmp) inactivated by ultra-violet light produced no higher mortality than normal allantoic fluid. Sixteen of 28 injected embryos were alive and normal when examined on the 11th day of incubation. We conclude that the lethal effect must be attributed to the presence of living virus.

Six embryos (of series 18mp) which were alive when recovered on the 4th day after infection were used for titration in 8-day embryos, the dilutions ranging from 10^{-2} to 10^{-7}. After 7 days' incubation hemagglutination showed the titer to have been 10^{-6}. Furthermore, the allantoic fluid and the emulsified tissues of the 6 original embryos of 18mp were tested by hemagglutination. The allantoic fluid titered to 1:32, the tissues were negative. The latter results correspond to those of Habel[7] where the allantoic fluids titered much higher than the tissue emulsions. Since it is known that mumps virus contained in allantoic fluid in a liquid state is completely inactivated within 48 hours at room temperature, we conclude that virus multiplication has taken place in our experiments.

The results obtained with mumps virus are of interest in two respects. First, Habel[7] had found that the infection of 8-day embryos has no lethal effect, although a virus multiplication in the allantoic sac can be demonstrated. The 2-day embryos seem to be more sensitive than the 8-day embryos since an infection is invariably lethal in the former. Second, the incidence of malformations is low, and there is no indication of the occurrence of specific gross abnormalities. Unless the histological examination of the material should reveal lesions of inner organs the mumps virus must be considered as a lethal but not a teratogenetic agent.

Summary. Experimental evidence has been presented to show that influenza-A virus (PR8) has teratogenetic effects on the early chick embryo. It produces a specific syndrome, comprising microcephaly and micrencephaly, twist of the axis and impairment of the growth of the amnion. Furthermore, the virus is lethal for early embryos, within 3 days after infection. The mumps virus is likewise lethal for early embryos, within 5 days after infection. It does not produce specific abnormalities but seems to raise the incidence of malformations of the types which occur occasionally in uninfected chick embryos. These results place Influenza-A virus in line with rubella virus, as a teratogenetic agent. Furthermore, our observations on Influenza-A infections in chick embryos confirm the observations on rubella in humans in that only infections of early embryos result in abnormalities. Chick embryos of 4 days of incubation are killed by the influenza virus, but it seems that at this stage of development most organs have passed the critical period at which their morphogenesis can be directed into atypical channels. In this respect, it is

of interest to find that the patterns of infectiousness are different for the embryo and for the fully developed structures. In the embryo, the brain tissues seem to be particularly susceptible to Influenza-A virus, whereas in the adult the respiratory mucous membranes are primarily affected. In mumps, the infection of the salivary glands is not infrequently combined with meningitis, but no effect on the brain was found macroscopically in embryos. The situation is the same as in rubella where the embryonic defects seem to have no obvious relations to the manifestations of rubella infections in older phases of life.

<div align="center">16173</div>

A Rapid Method for Demonstrating the Identity of Streptomycin-Producing Strains of *Streptomyces griseus*.[*][†]

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In a search for new strains of the streptomycin-producing organism, *Streptomyces griseus*, it is usually necessary to make a large number of isolations from the soil or other natural substrates, test the ability of the organisms to inhibit the growth of various bacteria, grow them in liquid media favorable for the production of the antibiotic, isolate the latter from the medium, and establish its identity with streptomycin. Out of a hundred or more cultures of *S. griseus* thus isolated and tested,[1,2] only very few were found capable of producing streptomycin. Most of the cultures formed no antibiotic at all, whereas some produced other antibiotics, such as grisein.[3]

To overcome this difficulty, two procedures were adopted: 1. The use, for isolation purposes, of media containing streptomycin, so as to eliminate most of the bacteria and the great majority of actinomycetes that are sensitive to streptomycin. 2. The use of *S. griseus* actinophage for rapid spotting of the streptomycin-producing strains of *S. griseus*, since these were found[4] to be sensitive to this actinophage, whereas all other strains of *S. griseus* were resistant.

The results of the following experiment illustrate the use of these procedures. One gram samples of soil, cow manure, and compost materials were plated out, in dilutions of $1:10^4$, $1:10^5$, $1:10^6$, and $1:10^7$, with nutrient agar containing 25 μg of streptomycin per ml. The plates were incubated at 28°C for 5 days. Five actinomyces colonies were isolated from several of the plates and grown upon slants of glucose-asparagine agar. Good growth was obtained after 7 days' incubation.

The 5 cultures were streaked on nutrient agar plates and incubated for 24 hours; drops of actinophage, both undiluted and diluted 1:10, were placed upon some of the streaks. The plates were again incubated for 24 hours at 28°C, and examined; the streaks that were treated with actinophage showed inhibition of sporulation and lysis of the vegetative mycelium.

A duplicate set of plates was streaked with the freshly isolated cultures, incubated for 48 hours, and cross-streaked with four test bacteria.[5] The plates were incubated for an additional 48 hours, and the zones of bacterial

1 Waksman, S. A., Schatz, A., and Reynolds, D. M., *Ann. N. Y. Acad. Sci.*, 1946, **48**, 73.

2 Waksman, S. A., Reilly, H. C., and Johnstone, D. B., *J. Bact.*, 1946, **52**, 393.

3 Reynolds, D. M., Schatz, A., and Waksman, S. A., *Proc. Soc. Exp. Biol. and Med.*, 1947, **64**, 50.

4 Reilly, H. C., Harris, D. A., and Waksman, S. A., *J. Bact.*, 1947, **54**

TABLE I.
Zones of Inhibition of Test Bacteria by Freshly Isolated Strains of *S. griseus*, as Measured by
Agar-streak Method.
Zone of inhibition measured in millimeters.

Strain No.	*Escherichia coli*	*Bacillus mycoides*	*Staphylococcus aureus*	*Bacillus subtilis*
1	20	20	18	26
2	23	23	19	28
3	20	20	17	27
4	22	22	20	26
5	20	22	17	23

TABLE II.
Production of Streptomycin by Freshly Isolated
Strains and Stock Culture of *S. griseus*.
Streptomycin, μg/ml.

Strain No.	Days of incubation		
	3	5	7
1	30	120	93
2	42	63	60
3	55	132	128
4	40	54	54
5	43	108	96
3475	42	81	65

inhibition recorded. The results (Table I) show that all the cultures were able to inhibit the growth of both gram-positive and gram-negative bacteria, the type of spectra obtained being reminiscent of streptomycin.[6]

The 5 cultures were inoculated into flasks with meat extract-peptone-glucose medium commonly used for the production of streptomycin. For comparative purposes, an active streptomycin-producing strain of *S. griseus* (No. 3475) was also used. The flasks were

FIG. 1.
Use of actinophage for identifying streptomycin-producing *S. griseus* cultures.

placed in a shaking machine in the incubator at 28°C. The activity of the culture filtrates was determined after 3, 5 and 7 days incubation by the cup method against a streptomycin standard (Table II).

The results show that the freshly isolated cultures were all active producers of streptomycin. That the antibiotic formed by the unknown strains of S. griseus was streptomycin or at least a streptomycin-like substance is illustrated by the similarity in the antibiotic spectra of the different preparations.

To illustrate further the effect of phage upon the unknown strains, the cultures were streaked upon agar plates. These were incubated for 24 hours, metal rings placed upon a portion of the area of growth of the organisms, and a few drops of actinophage placed inside each ring. The plates were incubated another 24 hours, the rings removed, and the plates examined. The action of the phage is illustrated in Fig. 1. The effect of the phage

was exactly the same as that usually produced on the streptomycin-producing strains of S. griseus.

Summary. A method for the rapid isolation and identification of streptomycin-producing strains of Streptomyces griseus is described. The method consists in plating out various dilutions of material from natural substrates, using agar media to which 25 μg/ml streptomycin has been added. Colonies of actinomycetes developing on the plates are picked and transferred to agar slants. When the cultures have developed, they are used to make agar streaks on plates. The plates are incubated for 24 hours, and drops of actinophage placed upon some of the streaks. The destruction of the growth of the actinomyces will prove the identity of the culture with streptomycin-producing S. griseus. This identification can be confirmed by the use of the agar-streak method, using various test bacteria, the sensitivity of which to streptomycin is known. Finally the cultures are grown in media used for the production of streptomycin, and the antibiotic produced is compared with streptomycin.

5 Waksman, S. A., and Reilly, H. C., *Anal. Ed., Ind. and Eng. Chem.*, 1945, 17, 556.

6 Schatz, A., Bugie, E., and Waksman, S. A., PROC. SOC. EXP. BIOL. AND MED., 1944, 55, 66.

16174 P

Enhancement of Heterophile and Bacterial Agglutination Titers by Means of Serum Diluent.*

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Various workers have shown that Rh agglutination titers are enhanced, and the action of the so called "blocking" antibody inhibited by the use of serum, plasma or bovine albumin instead of saline as a diluent.[1-6] Recently Griffiths[7] has reported the inhibition of Brucella blocking antibodies when rabbit serum is used in place of saline as a diluent in agglutination tests. This paper is a preliminary report on the enhancement of heterophile and various bacterial agglutinin titers by use of serum diluent instead of physiological saline. Routine agglutination tests were done comparing saline and pooled human

* Supported in part by the Michael Reese Research Foundation.

1 Wiener, A. S., PROC. SOC. EXP. BIOL. AND MED., 1944, 56, 173.

2 Race, R. R., *Nature*, 1944, 153, 771.

3 Diamond, L. K., and Denton, R. L., *J. Lab. and Clin. Med.*, 1945, 30, 821.

4 Wiener, A. S., *J. Lab. and Clin. Med.*, 1945, 30, 662.

5 Levine, P., *Am. J. Clin. Path.*, 1946, 16, 597.

6 Levine, P., and Walker, R. K., *Science*, 1946, 103, 389.

7 Griffitts, J. J., *Pub. Health Rep.*, 1947, 62, 865.

or rabbit serum as a diluent. Titrations using plasma, ascitic fluid and 20% bovine albumin diluents were also carried out.

Methods. Heterophile agglutination tests were done according to the technic of Paul and Bunnell.[8] Serial twofold dilutions were made of the patient's serum in saline diluent, and equal parts of 1% sheep cells in saline were added to each tube. The tubes were shaken and incubated at 37°C for 2 hours. Next they were removed and kept overnight in the icebox at 5°C. The titer was read as the highest dilution showing distinct visible clumps of erythrocytes. The tests using human or rabbit serum diluent were carried out in an identical manner except that serum was used in place of saline. The serum diluent was inactivated by heating at 56°C for ½ hour and tested for the presence of heterophile antibodies prior to use. Only serum diluent having no heterophile agglutinins was used. The Davidsohn guinea pig kidney absorption test for infectious mononucleosis[9] was carried out in a similar manner with the patient's serum.

The bacterial agglutination tests were performed in the following manner: Serial twofold dilutions of the patient's serum previously inactivated by heating at 56°C were made both in saline and serum diluents having no agglutinins for the test organism. The serum diluent was heated at 56°C for ½ hour before use. Equal parts of various bacterial antigens suspended in saline and adjusted to a turbidity of nephelometer (McFarland) tube No. 3 were added to each tube. The tube mixtures were shaken and incubated at 56°C for 2 hours. They were then kept overnight in the icebox at 5°C and read the following morning. The following bacterial antigens were studied: typhoid, paratyphoid A and B, Shigella, Brucella and paracolon.

Results. Heterophile Agglutination. Typical heterophile agglutinin titrations comparing saline and serum diluents in individuals with

8 Paul, J. R., and Bunnell, W. W., *Am. J. Med. Sciences*, 1932, **183**, 90.

9 Davidsohn, I., *J. A. M. A.*, 1937, **108**, 289.

† We are indebted to Dr. Karl Singer of the Hematology Department of Michael Reese Hospital for hematological studies.

TABLE I.
Enhancement of Heterophile Agglutination Titers by Means of Serum Diluent.

Patient	State of serum	Agglutinin titer	
		Saline diluent	Serum diluent
Positive Clinical and Hematological Findings.			
EN	Un*	1:5,120	1:40,960
	Abs†	1:1,280	1:5,120
EM	Un	1:80	1:1,280
	Abs	1:20	1:320
MC	Un	1:80	1:640
	Abs	1:20	1:160
SK	Un	0	1:80
	Abs	0	1:20
LM	Un	1:320	1:2,560
	Abs	1:80	1:320
DD	Un	1:40	1:640
	Abs	1:20	1:160
SR	Un	1:160	1:1,280
	Abs	1:40	1:160
IM	Un	1:5,120	1:40,960
	Abs	1:1,280	1:5,120
FS	Un	1:80	1:640
	Abs	1:40	1:160
Negative Clinical and Hematological Findings.			
WW	Un	1:20	1:320
	Abs	0	0
WA	Un	0	0
	Abs	0	0
WM	Un	1:10	1:80
	Abs	0	0
HM	Un	1:10	1:160
	Abs	0	0
JG	Un	0	0
	Abs	0	0
CR	Un	0	0
	Abs	0	0
GN	Un	0	0
	Abs	0	0
DR	Un	0	0
	Abs	0	0
MM	Un	0	1:160
	Abs	0	0
GW	Un	0	1:320
	Abs	0	0

* Unabsorbed.
† Absorbed with guinea pig kidney suspension.

positive and negative clinical and hematological† findings for infectious mononucleosis are summarized in Table I. Human plasma, ascitic fluid, rabbit serum, and 20% bovine albumin diluents also significantly enhanced heterophile agglutination titers. As shown in Table I heterophile agglutinins were detected in the serum of one patient (SK) with positive clinical and hematological findings for infectious mononucleosis using serum diluent, although repeatedly negative results were obtained in the routine saline test. In another positive case (EM) significant titers of heter-

TABLE II.
Enhancement of Bacterial Agglutination Titers by Means of Serum Diluent.

Patient	Test antigen	Agglutinin titer	
		Saline diluent	Serum diluent
PN	Typhoid H	0	0
	,, O	1:320	1:2,560
	Paratyphoid A	1:80	1:160
	,, B	1:160	1:1,280
NL	Flexner V	1:80	1:640
	,, Y	1:160	1:640
	Sonne	0	0
	Schmitz	1:80	1:640
	Shiga	0	0
JC	Flexner V	1:320	1:5,120
EG	Brucella abortus	1:40	1:160
	,, melitensis	1:80	1:80
	,, suis	1:40	1:80
JB	Typhoid O	0	0
	,, H	1:320	1:1,280
	Paratyphoid A	0	0
	,, B	1:160	1:1,280
CE	Paracolon	0	1:320

ophile agglutinins in both unabsorbed and absorbed serum were detected 5 days earlier with serum diluent. The confirmatory value of the guinea pig kidney absorption test in diagnosis of infectious mononucleosis[9] is emphasized by the fact that serum diluent failed to enhance titers of absorbed serums in negative cases.

Bacterial Agglutination. Preliminary results showing the enhancement of various bacterial agglutinin titers by means of serum diluent are given in Table II. It was noted that clumps of agglutinated bacteria in serum diluent were more easily dispersed on vigorous shaking than in saline. A similar enhancement of titer was observed with plasma, ascitic fluid, rabbit serum and 20% bovine albumin diluents. It is of interest that agglutinins for a paracolon bacillus isolated from a paratyphoid-like infection were demonstrable only by means of serum diluent.[10]

Comment. We feel that our results together with those previously reported by others suggest that not only does serum diluent inhibit the action of blocking antibody in certain serum, but that it is probably a more favorable medium in which the agglutination reaction can occur. Perhaps the presence of agglutinins can be detected sooner in the course of an infection by the routine use of serum diluent.

Summary. Heterophile and various bacterial agglutination titers were significantly enhanced by the use of serum diluent instead of saline. Similar results were obtained with plasma, ascitic fluid and bovine albumin diluents.

[10] Milzer, A., unpublished studies.

16175

Effect of Estrogen on Plasma Vitamin A of Normal and Thyroidectomized Rabbits.*

MARTIN B. WILLIAMSON. (Introduced by F. W. Bernhart.)
From the Harvard Medical School and the Massachusetts Eye and Ear Infirmary, Boston.

The congenital malformation in the eyes of young rats produced by a severe maternal deficiency of vitamin A during pregnancy,[1,2]

[1] Warkany, J., and Schraffenberger, E., Proc. Soc. Exp. Biol. and Med., 1944, 57, 49.

[2] Jackson, B., and Kinsey, V. E., Am. J. Ophthalm., 1946, 29, 1234.

appears to have a strong resemblance to the fibroplasia which has been observed to occur spontaneously behind the lenses of some premature human infants.[3] Since it seems unlikely that this retrolental fibroplasia in humans is due directly to a maternal deprivation of vitamin A,[2] other influences which might cause

[3] Terry, T. L., Am. J. Ophthalm., 1942, 25, 1409; Arch. Ophthalm., 1943, 29, 36, 54.

	Thyroidectomized			Normal		
	No. of animals	Liver Vit. A I.U./g	Plasma Vit. A I.U. %	No. of animals	Liver Vit. A I.U./g	Plasma Vit. A I.U. %
Control	4	80	401	4	64	206
Estrogen treated	4	55	245	4	47	182

a general, or even a local, deprivation of vitamin A were sought.

During the course of pregnancy, the blood vitamin A level falls markedly and continuously so that an abnormally low level obtains at term. This low vitamin A level returns to approximately normal in about 24 hours after parturition.[4,5] The behavior of the blood vitamin A during pregnancy appears to be the converse of that reported for the blood and urine estrogen levels; the estrogen output increases to a very high level and then drops precipitously directly after parturition.[6,7] An earlier report has also suggested that estrogens may inhibit the growth-stimulating effect of carotene.[8]

It was considered to be of value, therefore, to establish whether a relationship exists between the administration of estrogens and the blood level of vitamin A. Since thyroid material has a marked influence on both of the above substances,[9,10] the role of the thyroid has been taken into account in this study.

Experimental. Young animals were used in order to reduce the effect of their own sex hormones to a minimum. Weanling, albino rabbits were maintained on a normal chow diet. The rabbits in Exp. 1 were all females from one litter and were thyroidectomized at 5½ weeks of age.

Two weeks after thyroidectomy, the intramuscular injection of estrogen (α estradiol benzoate in peanut oil[†]) was begun. An injection of 2,000 R.U. (0.33 mg) was given twice a day to each animal; one day a week only one dose was injected. Thus, 64,000 R.U. were given to each experimental animal over 17 days in Exp. 1, and 68,000 R.U. over 19 days in Exp. II. The animals used in the second experiment were not thyroidectomized but were started on the estrogen treatment when approximately 6 weeks old. These rabbits were from 2 litters, 4 being females, and were distributed equally between the "control" and "estrogen treated" groups.

A blood sample was obtained by heart puncture before the first injection of estradiol and at various intervals during the course of the experiment. Finally, the animals were killed and the livers removed. The plasma samples were analyzed for their vitamin A content by the micromethod of Bessey, *et al.*[11] Aliquots of the livers were digested in 5-10 volumes of alcoholic KOH (5N KOH in 60% ethanol) on a hot water bath for 30 minutes and were then extracted with a 1:1 mixture of xylene and kerosene. The vitamin A concentration was then determined by the same procedure as that used for the determination in the plasma.

4 Jackson, B., and Kinsey, V. E., private communication.

5 Goldberger, M. A., and Frank, R. T., *Am. J. Obstet. Gynecol.*, 1942, **43**, 865.

6 Cole, H. H., and Saunders, F. J., *Endocrinology*, 1935, **19**, 199; Smith, G., Smith, O. W., and Pincus, G., *Am. J. Physiol.*, 1938, **121**, 98; Cohen. S. L., Marrian, G. F., and Watson, M. C., *Lancet*, 1935, **228**, 674.

7 Lund, C. J., and Kimble, M. S., *Am. J. Obstet. Gynecol.*, 1943, **46**, 486.

8 von Euler, H., and Klussmann, E., *Arkiv Kemie, Mineral Geol.*, 1932, **11B**, No. 1; *C. A.*, 1934, **26**, 3827.

9 McDonald, M. R., Riddle, O., and Smith, G. C., *Endocrinology*, 1945, **37**, 23.

10 Greaves, J. D., and Smith, C. L. A., *Am. J. Physiol.*, 1936, **116**, 456.

11 Bessey, O. A., Lowry, O. H., Brock, M. J., and Lopez, J. A., *J. Biol. Chem.*, 1946, **166**, 177.

† Part of the estradiol benzoate used was kindly furnished by Dr. F. E. Houghton of the Ciba Pharmaceutical Products, Inc.
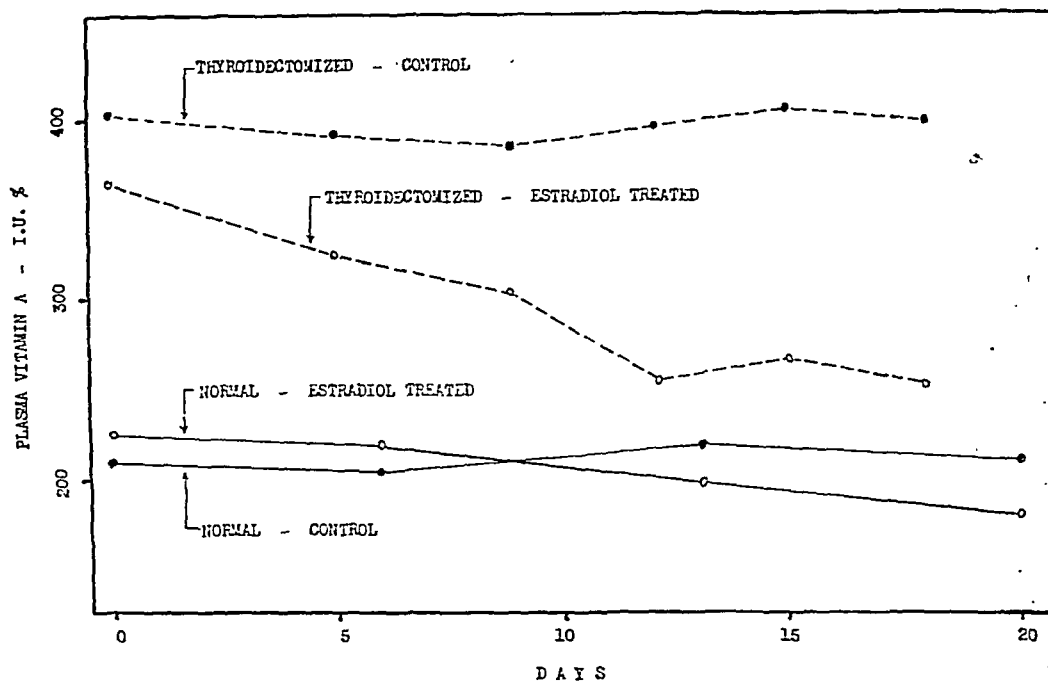
FIG. 1.
Plasma vitamin A as affected by the administration of α-estradiol benzoate. Broken line shows results obtained from thyroidectomized rabbits; solid line, from non-thyroidectomized normal animals.

The results of these experiments are shown in Fig. 1. The plasma vitamin A remained essentially unchanged in the control animals of both experiments. However, the level of vitamin A in the plasma of the estrogen treated rabbits showed a decline, particularly in the case of the thyroidectomized ones. The decrease observed in the non-thyroidectomized rabbits does not appear to be significant. It was found that the plasma level of vitamin A was higher in the thyroidectomized animals than in the normals. This higher level is probably due to the decreased metabolism of vitamin A observed after removal of the thyroid. The vitamin A content of the livers reflects the final plasma level determinations (Table I).

A possible explanation of the observed decrease in vitamin A may be found in the experiments of Loeb.[12] He reports that the

stores of body fat are increased under the influence of repeated estrogen injections. It would appear likely that the fat which is deposited may carry along some vitamin A, thereby lowering the plasma level.

The amount of estrogen used in these experiments was much greater than might be expected to be secreted by the pregnant animal. Yet, this large amount of estrogen did not depress the plasma vitamin A level below normal. It would then seem doubtful that the estrogens normally produced during pregnancy could influence the metabolism of vitamin A sufficiently to be considered a cause of the fibroplasia found in the eyes of premature infants.

Summary. Thyroidectomized and normal young rabbits were injected with large doses of estradiol benzoate for 17-19 days. The vitamin A level in the plasma of the thyroidectomized animals was significantly lowered. There was no appreciable decrease in the plasma vitamin A of the normal rabbits.

12 Loeb. H. G., PROC. SOC. EXP. BIOL. MED., 1942, 51, 330.

16176

Thyroid Hyperplasia in Rats Following Thymus Feeding.

K. KJERULF-JENSEN. (Introduced by H. Dam.)

From the Research Laboratory of Ferrosan Ltd., Copenhagen, Denmark

Hou[1] stated that rats fed on a soybean-millet diet with dried liver powder as the only source of animal protein developed a marked hypertrophy of the thyroid gland.* The thyroid hyperplasia did not seem to be due to administration of the animal protein as such, since rats given dried beef powder instead of liver powder developed only a relatively slight hyperplasia. The goitrogenic principle could be removed from the liver tissue by alcohol extraction. This pronounced goitrogenic effect of dried liver powder in Hou's experiments was so considerable that, according to our experience, feeding with about 0.1 mg 2-thio-4-methyl-uracil per 10 g diet would be required to produce a comparable enlargement of the thyroid gland.

The goitrogenic effect observed in the experiments reported below was not so pronounced as that seen in Hou's experiments. On the other hand, thyroid hyperplasia could be elicited not only by adding dried thymus powder to the soybean-millet diet, but also by giving a usual standard diet with addition of thymus. It was impossible in this case to remove the goitrogenic substance in the thymus tissue by alcohol extraction. Addition of corresponding amounts of dried ox-liver powder to the soybean-millet diet caused very little thyroid hyperplasia in these experiments. Dried beef powder had no goitrogenic effect. The goitrogenic effect of the thymus administered did not seem to be due to the contents of nucleoprotein as such, since no goiter was developed after administration of dried cod spawn in corresponding amounts.

Groups of 5 rats each were given the different diets. (The initial weights of the animals ranged from 60 to 120 g, so that the body weights by the end of the experimental periods

differed but little). The rats were killed after 30, 60, and 90 days respectively, and their thyroids were excised, weighed, and submitted to histological examination. The weights were indicated in percent of the normal, as pointed out in a previous paper by Jensen and Kjerulf-Jensen.[2]

The diet consisted of the following substances:

Soybean flour	450
Millet	500
Sodium chloride	20
Cod liver oil	1
Ascorbic acid	0.5

This diet was supplemented by

1. Dried thymus of young calves: 30 (1 g dried thymus corresponded to 4.7 g fresh thymus).

2. Residue of dried thymus gland, extracted by 5 vol. of ethyl alcohol at 25°C, in the above amounts.

3. Water-soluble fraction of the alcohol extract of dried thymus in the above amounts, as well as dried beef: 30.

4. Water-insoluble fraction of alcohol extract of dried thymus in the above amounts, as well as dried beef: 30.

5. Dried beef powder: 30.

6. Dried ox liver powder: 30.

7. Alcohol extract of dried ox liver in the above amounts, as well as dried beef: 30.

8. Dried cod spawn: 30.

The histological conditions were indicated as: $0 =$ normal, or: $+ =$ slight, *i.e.* slight cellular hyperplasia with increase in the height of the cells of the glandular epithelium, and inconsiderable reduction of the amount of colloid substance, or: $++$, *i.e.* increase in cell height as well as marked reduction of the amount of colloid substance.

Feeding with dried thymus elicited thyroid hyperplasia also in rats given the usual laboratory diet instead of the soybean-millet diet mentioned above. Administration of up to 10 times as much thymus added but little

1 Hou, C. H., PROC. SOC. EXP. BIOL. AND MED., 1940, 43, 753.

* For further references to literature *vide* the quoted article by Hou.

2 Jensen, K. A., and Kjerulf-Jensen, K., *Acta pharmacol.*, 1945, 1, 280.

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Diet No.	Avg size of thyroid in % of norm. for groups 5 rats for 30, 60, and 90 days respectively			Cellular hyperplasia of thyroid, estimated for each individual group as an entirety		
1. Dried thymus	123	130	126	+	++	++
2. Thymus, extr. by alcohol	120	124	136	++	++	++
3. Water soluble fract. of thymus alc. extr.	143	108	130	+	0	+
4. Water insolub. fract. of thymus alc. extr.	99	140	138	0	+	+
5. Dried beef	117	144	124	0	0	0
6. Dried liver	137	116	113	0	0	+
7. Alcohol extr. of ox liver	111	153	123	0	+	+
8. Dried cod spawn	115	106	108	0	0	0

to the increase in the size of the thyroid, whereas the histological changes, *i.e.* the cellular hyperplasia and especially the reduction of colloid substance became particularly pronounced.

Summary. Moderate cellular hyperplasia of the thyroid was produced in rats on a soy-bean-millet diet or a usual standard diet by adding dried thymus from calves.

The goitrogenic fraction was insoluble in water and ethyl alcohol; no goitrogenic effect was observed after feeding with dried ox liver, dried beef or dried cod spawn.

16177

Isolation and Properties of Raphanin, an Antibacterial Substance from Radish Seed.

G. Ivánovics and S. Horváth. (Introduced by G. Gomori.)

From the Institute of General Pathology and Bacteriology, University of Szeged, Hungary

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Experimental. The aqueous extract of the seeds is very effective in preventing the

growth of bacteria. The cell-free extract prepared by the extraction of one part of finely ground seeds with 5 parts of water gave a 30 mm zone of inhibition on plates seeded with *Staph. aureus* or *B. coli*. Even extracts prepared in the ratio of 1:50 caused marked inhibition. The antibacterial principle is fairly resistant to heat; only a slight decrease in potency was observed after heating it to the boiling point for 30 min. The cell-free extract will retain full activity for weeks if stored in the ice box. However, all activity is lost within 24 hours if the extract is incubated with the crushed seeds at 37°C. Considerable inactivation occurs under such conditions even at 20°C; in fact, even at ice box temperature. On the other hand, heating the suspension to 60°C for 15 min. completely prevents inactivation.

[1] Kavanagh, F., *Adv. Enzymology*, 1947, 7, 461.

[2] Ivánovics, G., and Horváth, S., *Nature*, 1947, 160, 297.

[3] Heatley, N. G., *Bioch. J.*, 1944, 38, 61.

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[3] Heatley, N. G., *Bioch. J.*, 1944, 38, 61.

TABLE I.

Extraction of Antibiotic by Various Solvents from Aqueous Extract of Seeds.

| | | Diameter of zone of inhibition in mm | | | | | |
| | | Dilution of organic solvent fraction | | | Dilution of aqueous residue | | |
Solvent	pH of extract	1:1	1:2	1:3	1:4	1:8	1:12
Amyl acetate	2	24	22	21	22	16	15
,, ,,	3	24	22	20	20	17	15
,, ,,	4	26	24	19	21	17	14
Butanol	3	27	26	26	16	14	8
Ethyl acetate	3	28	26	25	22	16	13
Petroleum ether	3	13	9	0	27	22	20

If intact or carefully peeled seeds are soaked in water for a few hours the diffusate is found to be devoid of activity, thus indicating that the active principle is firmly bound to the cells. Since the active substance obtained from crushed seeds passes a cellophane membrane with ease it is obvious that the seeds do not contain the active principle proper. This fact, together with the heat resistance of the active extract and with the failure to obtain active extracts from seeds boiled 15 min. before crushing them, led to the assumption that the seeds contain an enzyme capable of converting an inactive precursor into an antibiotic. The correctness of this assumption could be proven by the following experiment: Crushed seeds were extracted with 3 changes of 80% ethanol; the extract was concentrated and freed from alcohol *in vacuo* (extract A). Another portion of the crushed seeds was extracted with water and dialyzed in a cellophane bag against tap water (extract B). Both extracts were entirely devoid of any antibacterial activity. However, when the extracts were mixed, and the mixture allowed to stand for one hour, a potent solution resulted. No activity was obtained if extract B was heated to 70°C for 30 min. before adding it to extract A.

Crude extracts of the active principle were prepared as follows: 2000 g of finely ground seeds was stirred up with 3000 ml of tap water, and the mixture allowed to stand at room temperature for 30 to 40 min. The suspension was strained through cheesecloth, and the residue squeezed out in a hand press. The combined filtrates were adjusted to pH 5, and 14 to 15 ml of a 40% solution of basic lead acetate was added to each 100 ml of the juice. The precipitate was centrifuged off and discarded. The excess lead, which otherwise would have caused gradual inactivation, was removed by the addition of 7 to 8 ml of a 20% solution of $Na_2HPO_4 \cdot 12H_2O$. The supernatant gave only a very slight reaction with H_2S. It should be remarked here that the excess lead must not be removed with H_2S to which the active principle is sensitive. This procedure of purification caused a loss not exceeding 20%. The extract thus obtained will be referred to as the purified aqueous extract.

The active principle can be extracted with ease by various organic solvents at an acid pH. 40 ml of the purified aqueous extract was shaken with 20 ml of various organic solvents. The solvents were subsequently evaporated *in vacuo*, and the residue redissolved in 10 ml of water. Both the original aqueous phase and this latter solution were assayed by the cylinder plate method. The results are shown in Table I where the concentration of the redissolved organic solvent fraction is referred to as 1:1, while that of the original aqueous phase is taken as 1:4, in comparison to the other fraction.

The active principle is not absorbed on tricalcium phosphate or on kaolin. However, it is easily adsorbed on charcoal at pH 5 to 7. All attempts at elution between pH 2.2 and 11 failed.

In further experiments a butanol concentrate of the active principle served as a standard of reference. An amount producing a 19 to 21 mm wide zone of inhibition was chosen as an arbitrary unit of activity.

The pure active principle was isolated in

TABLE II
Elementary Composition of Raphanin and of Inactive Crystalline Substance.

Solvent used for extraction	Substance	% composition			
		C	H	N	S
Butanol	Raphanin, Batch 3				
	First fraction	41.71 ⎱ 41.27*	5.03 ⎱ 5.34*		
		41.10 ⎰	5.41 ⎰		
		41.00	5.58		
	Middle "	41.23 ⎱ 41.26*	5.28 ⎱ 5.18*	8.41	
		41.30 ⎰	5.08 ⎰		
	End "	41.66	5.27		
Butyl acetate	Raphanin, Batch 5†	41.62 ⎱ 41.50*	5.61 ⎱ 5.50*	8.91	33.94 ⎱ 33.64*
		41.38 ⎰	5.40 ⎰		33.34 ⎰
	Inact. cryst. substance	34.64	5.48	6.89	45.42 ⎱ 45.36*
		34.71 ⎱ 34.65*	5.09 ⎱ 5.31*	6.70 ⎱ 6.73	45.31 ⎰
		34.60 ⎰	5.35 ⎰	6.60 ⎰	
Theoretical values for	$C_{17}H_{26}N_3O_3S_5$	42.48	5.45	8.74	33.35
	$C_{17}H_{26}N_3O_4S_5$	42.11	5.28	8.58	33.03

* Average values
† Freshly redistilled by molecular distillation.

the following manner: 5330 ml of the purified aqueous extract containing 7370 units of the antibiotic was obtained from 2000 g of seeds. This solution was extracted with six 1000 ml portions of butyl acetate. The extracts were combined and assayed; total recovery was 5648 units. The solvent was removed by vacuum distillation. The resulting brown oily material was shaken with 200 ml of 0.07M phosphate buffer pH 7.2; the insoluble material was filtered off and discarded. The water-soluble fraction was extracted with 3 portions of chloroform, 30 ml each. The combined chloroform extracts contained 5500 units. The solution of the antibiotic in chloroform was shaken with 100 ml of phosphate buffer to remove the last traces of acidic impurities. The active principle, together with most of the pigment, remained in the chloroform layer. The solution was now passed through a column of Brockman's alumina which adsorbed a brick-red pigment. The percolate contained all the activity. After evaporation of the chloroform and of the last traces of moisture, a slightly yellow liquid of syrupy consistency was obtained. It had a sharp boiling point and could be distilled under reduced pressure without much decomposition. Boiling point: 135°C at 0.06 mm; 142.5°C at 0.09 mm, and 145°C at 0.1 mm Hg. Total yield, 7.5 g.
The distillate which consisted of the pure active principle was designated as raphanin. The homogeneity of the distillate could be established by redistillation at 135°C under a pressure of 0.06 mm Hg. Fractions were collected separately at intervals at the beginning, the middle and the end of the procedure and analyzed for C, H, N and S (Table II).

Raphanin is only moderately soluble in ether. The partition coefficient between butanol and water is favorable but butanol extracts contain a large amount of impurities which can be removed only with difficulty.

In an effort to reduce the amount of solvent required for extraction, the purified aqueous extract was concentrated *in vacuo* at 60°C to about one-half of its original volume. In one experiment 4500 ml of purified aqueous extract was reduced to 2000 ml. This operation resulted in the loss of about 1000 units. The concentrated liquid was extracted with butyl acetate; the solvent was evaporated *in vacuo* to a small volume. This concentrate was placed in the refrigerator, and after standing there overnight, a crop of crystals appeared. The crystals were collected, recrystallized first from boiling water, then from ethanol. Yield, 400 mg M. P. 192°C (decomp.). The elementary analysis is shown in Table II. This crystalline substance did not possess any antibacterial activity.

Some physical and chemical properties of raphanin. Freshly distilled raphanin has a

TABLE III.
Highest Dilutions of Raphanin Causing Inhibition of Bacterial Growth.

Organism	Medium	Partial inhibition × 1000	Complete inhibition × 1000
Staph. aureus	broth	1:50	1:28
,, ,,	serum broth*	1:1	1:1
,, ,,	casein hydr. broth†	1:50	1:28
B. coli	broth	1:10	1:5
,, ,,	serum broth*	1:1	1:1
,, ,,	synthetic‡	1:125	1:70
Salm. schottmülleri	broth	1:8	1:5
B. anthracis	,,	1:8	1:4
Pseud. aeruginosa	,,	1:16	1:8
,, ,,	serum broth*	1:4	1:2
Salm. typhi	broth	1:16	1:8
,, ,,	serum broth*	1:4	1:2

* Containing 50% ox serum.
† According to Ivánovics, Acta Med. Szeged, 1944, 12, fasc. 1.
‡ According to Sahyun, Beard, Schultz, Snow, and Cross, J. Infect. Dis., 1936, 58, 58.

TABLE IV.
Zones of Inhibition in mm with Various Concentrations of Raphanin.

Organism	Zone of inhibition in mm		
	1 mg/ml	2 mg/ml	3 mg/ml
Staph. aureus	18.5	22.5	24
B. anthracis (avirulent)	23	26.5	28
,, ,, (virulent)	22	26.5	29
,, subtilis (strain Duthie)	20	26	29
,, coli	16	19.5	21
Salm. typhi—H	19	24.5	27
,, ,, —O	24	26.5	28.5
,, schottmülleri	22	28.5	31
B. shiga-kruse	16	20	22
Pseud. aeruginosa	19	23	25
B. prodigiosus	12	15	19

slightly yellowish shade which will darken on storage at room temperature. It was attempted to obtain a colorless sample by molecular distillation at 60°C. The distillate had a very minimal yellowish tinge; it was impossible to secure an entirely colorless preparation. It is believed that the yellowish shade is due to decomposition products although darkening at room temperature is not associated with any noticeable loss in antibacterial activity.

The substance has a radish-like odor. It is quite soluble in water with a neutral reaction. Its solution in absolute ethanol is levorotatory; $[\alpha]_D^{20} = -141°$. Freshly prepared aqueous solutions give no coloration with ferric chloride or with nitroprusside. However, after treatment with dilute HCl and Zn dust, an intense purple shade was obtained with nitroprusside, indicating the presence of –SH groups. If the aqueous solution is heated with Ag or Pb salts, a heavy black precipitate, insoluble in HNO_3, is obtained, and the supernatant is found to have lost its antibacterial activity.

If raphanin is shaken with 0.1N Ba(OH)$_2$ at room temperature, the excess alkali neutralized with H_2SO_4, the $BaSO_4$ precipitate removed by filtration, and the filtrate, evaporated to a small volume, is placed in the refrigerator, crystals will precipitate on standing. This crystalline substance is inactive. M.P., after recrystallization from ethanol, is 192-93°C (decomp.). When these crystals were mixed with those obtained from the butyl acetate concentrate, no depression of the melting point was observed.

Antibacterial activity of raphanin. Raphanin was assayed on a number of bacterial species, using both the serial dilution method

TABLE V.

Residual Antibacterial Activity of Raphanin After Heating Its Solutions for 30 min. at Different pH (expressed in per cents of original activity).

Buffer	pH	Temperature			
		20	60	80	100°C
Phosphate	5.3	100	100	85	40
,,	7.2	100	80	50	0
Glycine	8.0	90	75	50	0
,,	10	50	0	0	
,,	11	40	0	0	

and the cylinder plate technique. An aqueous solution of freshly distilled raphanin was sterilized by filtration through a fritted glass plate. Various amounts of raphanin were added to 4 ml of nutrient medium, and the volume was made up with dist. water to 5 ml. The tubes were inoculated with 0.1 ml of a dilute bacterium suspension containing about 50,000 organisms in one ml. They were incubated for 24 hours at 37°C. (Table III). In tests by the cylinder plate method 3 different dilutions containing 1, 2 and 3 mg of raphanin per ml were used (Table IV).

The activity of raphanin against *B. coli* is superior to that of crystalline penicillin G since a 1:1000 dilution of raphanin gave a zone of inhibition of 18 mm, whereas penicillin in the same dilution gave a zone of only 13 mm. Even a 1:5000 dilution of raphanin produced noticeable inhibition while penicillin in a strength of 1:3000 was without effect.

Raphanin is readily inactivated in the alkaline range while it is much more stable at a neutral or slightly acid solution. 25 mg of raphanin was dissolved in 5 ml of buffer solution; the solutions were incubated at various temperatures for 30 min, and the activity was tested by the cylinder-plate method (Table V).

As mentioned, raphanin is rapidly inactivated by H_2S. However, this activation is dependent on the pH of the medium. If H_2S is bubbled through a buffered solution of raphanin for 3 min, and the gas subsequently removed by aeration, complete inactivation occurs at pH 7.2, but only 50% inactivation at pH 5.

Toxicity of raphanin. Owing to an acute shortage of experimental animals, the toxicity of raphanin could be tested only in a small number of mice and guinea pigs. Mice weighing 16 to 22 g were injected i.v. with doses of raphanin ranging from 5 to 40 mg. Five mg caused only transitory weakness, excitement and ruffled fur, and the animals recovered promptly. Doses of 7 to 10 mg killed the animals within 5 to 10 min, while doses of 20 to 40 mg caused death in a few seconds. The results were very similar when raphanin was injected subcutaneously, except that the appearance of the symptoms was somewhat delayed. The symptoms after the injection of a lethal dose were weakness, restlessness, dyspnea and clonic convulsions. Animals injected with 10 to 20 mg scratched their noses furiously with their hind legs. At post-mortem examination no gross anatomical changes were found, except for a slight edema at the site of injection.

Three guinea pigs, weighing 450 to 470 g, were injected intracardially with 25, 50 and 125 mg. No ill effect was observed after 25 mg; 50 mg caused death after 30 min, while the injection of 125 mg was followed by signs of weakness and excitement, and the animal died in convulsions after a few minutes.

In tissue cultures of rabbit testis, a 20,000 dilution of raphanin completely prevented the growth of fibroblasts; there was a definite inhibition of growth at a dilution of 1:40,000, while a dilution of 1:80,000 did not cause any inhibition.

Raphanin is a potent inhibitor of the germination of various plant seeds, including those of radish itself. Seeds were placed on discs of filter paper soaked in dilute solutions of the antibiotic. The discs were kept in a moist chamber for 3 days at room temperature. Results are shown in Table VI.

TABLE VI.
The Germination of Seeds of Different Plants in the Presence of Raphanin.

Plant species	Conc. of raphanin	No. of seeds	No. of seeds germinated	Avg length of roots in mm
Raphanus sativus	0 (control)	25	23	21.5
,, ,,	1:200	20	1	3.0
,, ,,	1:1,000	25	24	14.0
,, ,,	1:10,000	25	25	20.0
Brassica oleracea	0	25	20	17.2
,, ,,	1:1,000	25	8	4.0
,, ,,	1:10,000	25	20	11.0
Festuca pratensis	0	20	14	8.6
,, ,,	1:1,000	20	1	4.0
,, ,,	1:10,000	20	13	5.3
Sinapis alba	0	25	19	10.2
,, ,,	1:1,000	25	0	0
,, ,,	1:10,000	25	12	12.5
Hordeum distichon	0	20	20	16.0
,, ,,	1:1,000	20	6	7.7
,, ,,	1:10,000	20	20	12.8

Comments. The molecular weight of raphanin has not been estimated yet but on the basis of data of the elementary analysis the empirical formula could be either $C_{17}H_{26}O_3N_3S_5$ or $C_{17}H_{26}O_4N_3S_5$. The negative nitroprusside reaction indicates the absence of –SH groups and rules out the existence of an unsaturated lactone ring in the molecule. On the other hand, the appearance of –SH groups after reduction with nascent hydrogen is suggestive of a disulfide linkage.

The seeds of some of the Cruciferae contain thioglucosides which are hydrolyzed by concomitant enzymes into sugars and aglycons, the latter being mustard oils (isothiocyanic esters). This fact is of importance because it is asserted[4] that allyl isothiocyanate and related mustard oils possess an antibacterial action, a statement not borne out by other observations.[5] We were also unable to demonstrate any antibiotic effect in the seeds of *Sinapis alba*. Raphanin, as can be judged from its composition and chemical behavior,

does not seem to belong in the group of mustard oils, although data so far obtained do not permit any definite conclusions as to its exact structure. It bears some resemblance to gliotoxin isolated from *Aspergillus fumigatus* and to allicin. Both of these latter antibiotics contain a high percentage of sulfur in a disulfide linkage; both are effective against Gram-positive as well as against Gram-negative organisms, and both are rather toxic. It is interesting to note that the intact cells of garlic do not contain allicin but only an inactive precursor which is transformed by a concomitant enzyme into active allicin.[6]

Summary. The preparation and purification of a new antibiotic, raphanin, from the seeds of the radish is described. The seeds contain an inactive precursor which is activated to a potent antibiotic by a concomitant enzyme.

The physical, chemical, antibiotic and toxic properties of raphanin are described. Raphanin, owing to its high toxicity, does not hold the promise of a useful therapeutic agent.

4 Foter, M. J., and Golick, A. M., *Food Res.*, 1938, **3**, 609.

5 Osborn, E. M., *Brit. J. Exp. Path.*, 1943, **24**, 227.

6 Cavallito, C. J., Bailey, J. H., and Kircher, F. K., *J. Am. Chem. Soc.*, 1944, **66**, 1950.

16178

Chronic Riboflavin Deficiency in the Rat. I. Ossification in the Proximal Tibial Epiphysis.[*]

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The importance of riboflavin in skeletal development has been recently emphasized by Warkany.[1] Warkany found that prenatal riboflavin deficiency in the rat resulted in the production of young with multiple skeletal abnormalities. Among the abnormalities were developmental disturbances of the mandible, shortening and distortion of the extremities, various forms of syndactyly of the hands and feet, and fusion of the ribs and sternal centers of ossification. Histologically, a more or less marked delay in ossification occurred with persistence of cartilage in many areas. Levy and Silberberg[2] recently reported that in the mouse deficiency of riboflavin from weaning resulted in retardation of endochondral ossification and increased "degeneration of cartilage."

In the present study the changes in endochondral ossification in the tibia that occurred in rats born from riboflavin-deficient mothers and maintaind for three to nine months on purified diets deficient in riboflavin are reported. The riboflavin deficiency produced under these experimental conditons was chronic in nature.

Experimental. Normal female rats of the Long-Evans strain, 2 to 3 months of age, were bred with normal males and placed on riboflavin-deficient or control diets the day of breeding. At birth the riboflavin-deficient young were carefully examined for skeletal abormalities. All litters[†] were weighed every 5 days, weaned on the twenty-first day and continued on the same diet as their parents.

Previous studies[3,4,5] have disclosed that riboflavin deficiency is accentuated by a high fat diet. For this reason 2 riboflavin-deficient diets were used; one was high in carbohydrate and the other, high in fat. The latter was given from 90 days of age to the end of the experiment. The high-carbohydrate diet was composed of 24% alcohol-extracted casein, 64% sucrose, 8% hydrogenated cottonseed oil (Crisco), and 4% salts No. 4.[6] The high-fat diet contained 48% instead of 8% hydrogenated cottonseed oil with a corresponding reduction in the proportion of sucrose. Both diets contained the following crystalline B vitamins per kilogram of diet: 5 mg thiamine HCl, 5 mg pyridoxine HCl, 10 mg p-aminobenzoic acid, 20 mg nicotinic acid, 50 mg calcium pantothenate, 400 mg inositol, and one gram choline chloride. A fat-soluble vitamin mixture was given weekly and contained 325 mg corn oil (Mazola), 400 U.S.P. units vitamin A, 58 Chick Units vitamin D, and 3 mg alpha-tocopherol. During the lactation period the lactating mothers received a double

1 Warkany, J., *Vitamins and Hormones*, 1945, 3, 73, Academic Press. Inc., New York, Publishers.

2 Levy, B. M., and Silberberg, R., Proc. Soc. Exp. Biol. and Med., 1946, 63, 355.

† Six young were kept per litter.

3 Mannering, G. J., Lipton, M. A., Elvehjem, C. A., and Hart, E. B., Proc. Soc. Exp. Biol. and Med., 1941, 46, 100.

4 Mannering, G. J., Orsini, D., and Elvehjem, C. A., *J. Nutrition*, 1944, 28, 141.

5 Czackes, J. W., and Guggenheim, K., *J. Biol. Chem.*, 1946, 162, 267.

6 Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart. E. B., *J. Biol. Chem.*, 1941, 138, 459.

TABLE I.
Riboflavin Deficiency in Male Rats from Birth.

| Group | Animal No. | At autopsy | | Length of tibia* (mm) | Width of proximal epiphyseal cartilage of tibia† (μ) |
		Age (day)	Wt (g)		
I Riboflavin control group	B8492	96	317	39.2	244
	BH8494	110	352	41.2	165
	BH8487	144	436	38.2　Avg　40.5	163　Avg　143
	B7985	194	537	41.8	130
	GH7980	239	520	39.6	144
	BH7986	278	630	42.9	110
II Riboflavin deficient group	B6491	96	41	24.1	124
	GH2467	110	94	32.7	114
	B2498	144	122	35.0　Avg　34.3	114　Avg　111
	BH2499	194	169	37.9	123
	B2495	237	127	36.9	100
	B2493	277	188	39.3	89

* The length of the tibia was measured with calipers before decalcification.
† As measured in the central portion of histologic sections with an ocular micrometer.

amount of the fat-soluble vitamin allotment. Control rats received the identical purified diet supplemented with 10 mg riboflavin per kilogram of diet.

For the histologic study of the tibia 6 riboflavin-deficient animals that were most severely affected as judged by external appearance and retardation of body weight were selected. These animals together with the riboflavin controls were autopsied at ages varying from 3 to 9 months, *i.e.* 96, 110, 144, 194, 237 and 278 days (Table I). The tibias were fixed in 10% neutral formol, roentgenographed and measured, decalcified in 5% nitric acid, embedded in nitrocellulose, sectioned at 8-10 micra, and stained with hematoxylin and eosin.

Results. During the first 90 days of the experiment on the high-carbohydrate diet, only a mild riboflavin deficiency was produced, the rats showing few deficiency symptoms other than retardation of growth. In agreement with the findings of previous investigators, the deficiency was accentuated by the high-fat diet. Dermatitis, alopecia, cataracts, spasticity, and marked decreases in growth occurred in many animals. However, the prolonged survival of many animals (*see* autopsy ages, Table I) showed that the deficiency was chronic rather than acute even with the high-fat diet. It may be mentioned that no skeletal abnormalities were observed in the young born from riboflavin-deficient

mothers given either the high-carbohydrate or high-fat diets the day of breeding. Neither were abnormalities found in the second litters produced by these same mothers after they had received the deficient diets for 50-60 days at the time of the second breeding.

In comparing the tibial length and width of the epiphyseal discs marked differences were found between the riboflavin-deficient and the control group (Table I). The average length in the riboflavin-deficient group was 34.4 mm against 40.5 mm in the riboflavin-control group. Epiphyseal disc measurements averaged 111 micra for the deficient as compared with 143 micra for the control animals. Thus the decrease in epiphyseal disc width was aligned with shorter tibial length. The greatest changes in tibial length and cartilage disc width occurred in the youngest deficient animals. Severe decreases in body weight were found simultaneously (Table I).

The histologic aspect of the epiphyseal cartilage of the tibia in normal rats and changes occurring with increasing age have been recently presented in detail.[7] In this study measurements of tibial length and epiphyseal disc width of the riboflavin control animals were slightly but consistently below normal at every age, although histologic differences were slight. A microscopic study of the process of endochondral ossification in the tibia revealed decided differences between the
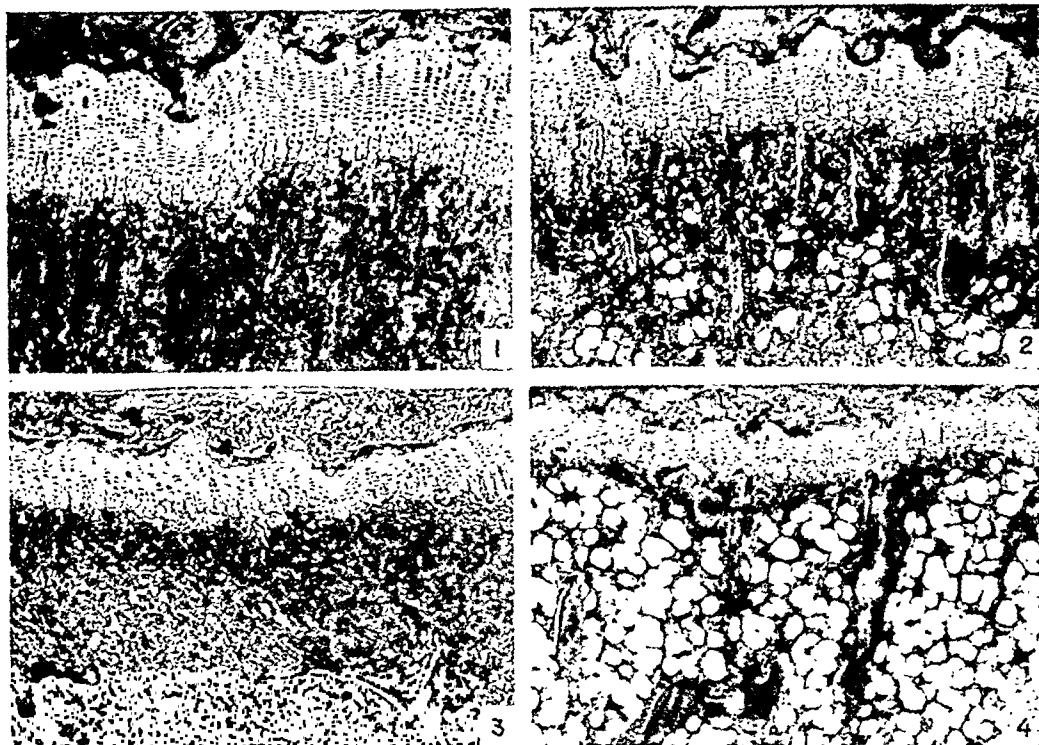
Proximal epiphyseal region of the tibia of male rats, H. and E. stain, × 75.
FIG. 1.   Riboflavin control, 96 days of age. Spec. No. 8908, Plate 9426.
F.G. 2.   Riboflavin control, 144 days of age. Spec. No. 8912, Plate 9427.
FIG. 3.   Riboflavin-deficient rat, 96 days of age. Spec. No. 8896, Plate 9431.
FIG. 4.   Riboflavin-deficient rat, 144 days of age. Spec. No. 8900, Plate 9429.

riboflavin-deficient and the control group.

Riboflavin Control Group. At 96 and 110 days the histologic aspect of the proximal epiphysis shows the normally thick surface cartilage, dense lamellar coat of bone, numerous coarse trabeculae, and a continuous layer of bone resting against the cartilaginous plate. The epiphyseal cartilage and subjacent diaphyseal area of the riboflavin control animal at 96 days of age are illustrated in Fig. 1. The trabeculae are numerous, close and coarse but do not extend far into the diaphysis. Few anastomoses are seen and erosion is very active. At 144 days (Fig. 2) the epiphyseal cartilage of the control animal shows a reduction in width, an increase of matrix and a more pronounced conical pattern of chondrocytes than usual for this age. The bone trabeculae are shorter and coarser and in many areas the marrow tissue is found in

direct contact with the zone of enlarged cells. Many osteoclasts are seen on the surface of the bone trabeculae. In the more advanced age groups at 194, 237, and 278 days of age, the cartilaginous plate became progressively narrower but richer in matrix and the columnar arrangement of cells appeared still more irregular and frequently lost even the Dawsonian pyramidal aspect of normal old age. The bone trabeculae disappeared for the most part except for a few lying horizontally some distance below the disc. As contrasted with animals reared on a diet of natural foods[7] ossification was subnormal and trabeculation somewhat disturbed.

Riboflavin Deficient Group. At 96 days the epiphyseal surface cartilage was well-formed

[7] Beeks, H., Simpson, M. E., and Evans, H. M., *Anat. Rec.*, 1945, 92, 109.

but the adjacent lamina of bone was so delicate as to be almost non-existent. The trabeculae were sparse in number and fine in structure. In the epiphysis, there was a thin, nearly continuous, horizontal layer of lamellar bone resting directly upon the cartilage (Fig. 3). The cartilaginous epiphyseal disc consists of 5 to 9 flattened cells and only one to 2 vesicular cells. The matrix is abundant in the basophilic region with bands occasionally traversing the entire width of the plate. Calcification of the matrix from the diaphyseal side is in progress and is enclosing the majority of vesicular cells. Osteogenesis has practically ceased and trabeculation consists only of short projections covered with true bone. This stage foreshadows the sealing-off of the epiphyseal cartilage from the marrow cavity by bone. Erosion is rare. The marrow shows an unusual wealth of osteoclasts but is normal in fat content.

At 110 days the epiphysis was somewhat larger; otherwise the appearance was the same as that of the 96-day-old tibia. The cartilage disc was slightly reduced in width. There was no evidence of fat infiltration in the marrow.

At 144 days the changes were more severe. The epiphysis showed advanced ossification. The bone lamina beneath the articulating cartilage, the trabeculae, and the layer of bone above the epiphyseal disc were increased in thickness. The cartilaginous disc (Fig. 4) is very narrow and the chondrocytes in the basophilic zone are arranged conically. The vesicular cells are enclosed in calcified matrix having a well-advanced marginal deposit of lamellar bone sealing it from the marrow. Three or 4 heavy isolated trabeculae extend into the diaphysis. Numerous osteoclasts are present in the marrow cavity and the number of large fat cells is increased markedly.

The findings in the tibias of the older age groups were similar. The margins of the epiphyseal cartilage were highly irregular and the matrix between the pyramidal groups of cells was increased. At 277 days the cartilage disc was the narrowest observed in this experiment and was reduced in a few places to 40 micra. The matrix lay in wide tracts in vertical or oblique planes across the disc, often

penetrating the marrow on the diaphyseal side and forming short trabeculae which showed lamellar bone on their surface. Much of the disc was separated from the marrow by true bone. The 3 or 4 remaining lamellar trabeculae evinced no particular 'orientation with respect to the diaphysis. The marrow contained an unreduced amount of fat cells.

Discussion. The foregoing experiment was planned to study the effects of prenatal riboflavin deficiency on skeletal growth in the young. In contrast to Warkany, no skeletal abnormalities were observed at birth and the animals were sacrificed at ages varying from 3 to 9 months. The pathological changes observed were not necessarily progressive with age since the animals had varying degrees of the deficiency at the time of autopsy. Some of the older animals had lived considerably longer before showing outward signs of the deficiency and were less affected than animals autopsied at an earlier age. Also histological changes in the tibia due to age may have masked some of the changes due to riboflavin deficiency in the older animals. However, even at 278 days, the tibia of the deficient animal, although showing the characteristic changes of advanced age, differed markedly from that of the riboflavin control.

The principal histologic changes observed in the riboflavin-deficient group were retarded development of the epiphysis, progressive decrease in the width of the epiphyseal cartilage, increased hyalinization of its matrix, calcification and separation of the epiphyseal cartilage from the marrow cavity by a thin layer of bone. Osteogenesis ceased with an almost complete disappearance of the diaphyseal trabeculae. Unusual fat infiltration with reduction of hematopoietic tissue occurred in most of the older animals and was similar to the myeloid hypoplasia recently reported by Endicott *et al.*[‡,8] for acute riboflavin de-

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‡ Endicott *et alia*[8] also found that both riboflavin and "folic acid" were concerned in this hypoplasia. Further studies will be necessary to separate the effects of these two vitamins on endochondral ossification.

8 Endicott, K. M., Kornberg, A., and Ott, M., *Blood*, 1947, **2**, 164.

ficiency in the rat. In general, the changes observed in this study of the rat were similar but much more severe than those reported for the mouse by Levy and Silberberg.[2]

Reasons for the failure to duplicate the Warkany findings (*i.e.* multiple skeletal abnormalities) are not obvious. The experimental conditions and the purified high-carbohydrate diet were similar but not identical with those used by Warkany. The principal dietary difference was the use of an alcohol-extracted casein, probably containing slightly more riboflavin[9] than that used by Warkany, at a 24% instead of 18% level in the diet. The use of a high-fat diet to accentuate the deficiency should have overcome this difference in the protein components of the diets. At the present time 40 litters comprising 350 riboflavin-deficient young have been carefully examined at birth. While clearing with microscopic examination might have revealed some skeletal defects, abnormalities such as shortening of the mandible with protrusion

of the tongue and syndactylism of the extremities which occurred with high frequency in Warkany's abnormal young could have been easily recognized by external inspection. Further studies on this problem are in progress.

Summary. The tibias of 6 male rats of the Long-Evans strain born from riboflavin deficient mothers and maintained on riboflavin deficient diets for 3 to 9 months were studied histologically and compared with an equal number of controls reared upon the same diet but with riboflavin added.

In the deficient rats the growth of the tibia was retarded and endochondral ossification was gravely impaired. Marked reduction in chondrogenesis and in capillary erosion and ossification were characteristic for all degrees of the deficiency. The formation of a thin calcified plate on the diaphyseal side of the epiphyseal cartilage is a finding in consonance with the early arrest of growth. Hematopoietic tissue was replaced by fat in all animals after 144 days on the deficient diet.

9 Cannon, M. D., Boutwell, R. K., and Elvehjem, C. A., *Science*, 1945, **102**, 529.

16179

A New Method for the Production of Non-Specific Capsular Swelling of the Pneumococcus.

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Neufeld,[1] and Neufeld and Etinger-Tulczynska[2] first described the phenomenon of capsular swelling of the pneumococcus when specific antiserum was added to the organism. This capsular swelling, known as the "quellung" reaction, is dependent upon the reaction of capsular polysaccharide with its specific antibody. It has been shown that

an excess of specific polysaccharide,[3] heat,[4] papain digestion,[5] and repeated washing with water[6] can produce a reversal of the specific "quellung" reaction.

Non-specific capsular swelling of pneumo-

3 Nungester, W. J., and Kempf, A. H., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 705.

4 Etinger-Tulczynska, R., *Z. f. Hyg. u. Infectionskrankh.*, 1933, **114**, 769.

5 Kalmanson, G. M., and Bronfenbrenner, J., *Science*, 1942, **96**, 21.

6 Kempf, A. H., and Nungester, W. J., *J. Inf. Dis.*, 1942, **71**, 50.

1 Neufeld, F., *Z. f. Hyg. u. Infectionskrankh.*, 1902, **40**, 54.

2 Neufeld, F., and Etinger-Tulczynska, R., *Ibid.*, 1931, **112**, 492.

TABLE I.

The Effect of Various Proteins and Non-Protein Substances on Capsular Swelling of the Type 27 Pneumococcus.

Substance tested	pH 4.0	pH 5.0	pH 5.5	pH 6.0
Crystalline urease	4+*	0	0	0
Diaphorase	2+	0	0	0
3-Phosphoglyceraldehyde dehydrogenase	0	3+	4+	2+
Purified horse serum albumin	3+	0	0	0
,, ,, hemoglobin	4+	0	0	0
Crude thrombin	4+	0	0	0
Zinc insulin	2+	0	0	0
Cytochrome C	0	0	0	0
,, ,, (dialyzed)	0	0	0	0
Egg albumin	4+	0	0	0
Boiled horse serum albumin	3+	0	0	0
,, ,, hemoglobin	4+	0	0	0
Commercial gelatin	1+	0	0	0
Wheat gluten	2+	0	0	0
Inositol	0	0	0	0
Inulin	0	0	0	0
Lactic acid, M/100	0	0	0	0

* Intensity of capsular swelling is indicated by the conventional notation from 0 to 4+ (maximum).

cocci was first reported by Lofstrom,[7] who used human sera obtained from patients in the acute phase of a febrile, usually bacterial, illness. When such sera were mixed with several different pneumococcal types, capsular swelling occurred. Type 27 pneumococcus was found to react most effectively. Similar acute-phase serum had been shown to produce a precipitate with "C" carbohydrate prepared from a rough strain of pneumococcus.[8-13] Both capsular swelling and the precipitin reaction are reversed by the addition of sodium citrate to remove ionized calcium.[12-14] Reciprocal-absorption experiments revealed that the same reactive protein produced a precipitate with "C" carbohydrate as produced pneumococcal capsular swelling.[13]

Recent work in this laboratory on the "non-specific capsular swelling" properties of

human sera for Type 27 pneumococcus has revealed another mechanism for the production of a "quellung" reaction. As far as the author can discover, this mechanism has not been previously described. In the course of experiments to determine the effect of pH on the "non-specific capsular swelling" phenomenon of Lofstrom, acute-phase serum was treated with 0.01 M lactic acid. At pH 5.0-5.5 a protein fraction was precipitated that was partially soluble in an 0.01 M lactic acid solution adjusted to pH 4.0. When formalin-killed Type 27 pneumococcus was added to this soluble fraction at pH 4.0, capsular swelling was observed. All of the human sera tested, normal or abnormal, displayed this reaction. Studies on beef, swine, and lamb serum revealed a similar reaction.

Purified preparations of several enzymes, horse-serum albumin, and horse hemoglobin* were found to react effectively to produce capsular swelling at pH 4.0 (Table I). Test of serial dilutions of the albumin and hemoglobin solutions revealed that the minimal amount of the proteins required to produce "quellung" of the pneumococci was 120 and

7 Lofstrom, G., *Acta Med. Scand.*, 1943, Suppl. 141, 1.

8 Tillet, W. S., and Francis, T., Jr., *J. Exp. Med.*, 1930, 52, 561.

9 Tillet, W. S., Goebel, W. F., and Avery, O. T., *J. Exp. Med.*, 1930, 52, 895.

10 Ash, R., *J. Inf. Dis.*, 1933, 53, 89.

11 Abernethy, T. J., and Avery, O. T., *J. Exp. Med.*, 1941, 73, 173.

12 MacLeod, C. M., and Avery, O. T., *J. Exp. Med.*, 1941, 73, 183.

13 Lofstrom, G., *Br. J. Exp. Path.*, 1944, 25, 21.

14 Carlens, E., *Acta Otolaryng.*, 1941, 29, 316.

20 μg per ml, respectively, when enough organisms were added to give one or 2 pneumococci per oil-immersion field. Non-protein substances and cytochrome C (Table I) were non-reactive. In distinction to the other materials tested, 3-phospho-glyceraldehyde-dehydrogenase was optimally reactive at pH 5.5.

Inhibition of the "quellung" reaction could be brought about by the addition of readily ionized salts to the protein-pneumococcus mixture adjusted to pH 4.0. As low a concentration as 0.02 M sodium chloride and 0.07 M potassium iodide were effective in inhibiting capsular swelling. On the other hand, the reaction was not inhibited in 0.55 M glucose.

When purified horse hemoglobin was used to produce capsular swelling, it was noted that condensation of the protein occurred around the enlarged capsule. This gave the appearance of a distinct rim of golden pigment dotting the periphery of the capsule. As the reaction proceeded, each pneumococcus was completely surrounded by hemoglobin. Eosin azo-dog serum, in a similar manner, produced capsular swelling. The protein appeared to penetrate the capsule to give it a diffusely stained, deep-pink color. When specific antiserum was added to maintain capsular swelling and the pH was brought back to 7.0, the pink-staining protein was no longer present in the capsule. Pneumococci were treated with eosin azo-protein at pH 4.0, centrifuged free of the solution, brought to pH 7.0 and then placed in a colorless protein solution at pH 4.0. These organisms then contained no microscopically visible eosin azo-protein in the swollen capsular substance. The reaction would appear to demonstrate a reversible combination of protein with the capsular polysaccharide, dependent on conditions of pH.

This new method of producing non-specific capsular swelling is not peculiar to Type 27 pneumococcus. Formalin-treated pneumococci, Types 1, 2, 3, 5, 7, 8, 19, and 28 were tested and all reacted with equal facility to several proteins. Similar capsular swelling did not occur, however, with *Klebsiella pneumoniae*, a mucoid *Escherichia coli*, or a mucoid *Strepto-*

coccus hemolyticus, although acid agglutination was observed under these conditions.

An attempt was made to determine the reactivity of purified polysaccharide substances with different non-specific proteins at pH 4.0. Both Type 28 and Type 3 polysaccharide substances were found to be reactive with proteins at pH 4.0 to produce a dense flocculent precipitate. This combination was reversible in that the precipitate washed in 0.01 M lactic acid solution adjusted to pH 4.0 and then brought to pH 7.0 was found to be clear and to react by precipitation with Type 3 specific antiserum. A pH of 4.0 was well below the iso-electric range of the proteins tested, and the pH of each protein-carbohydrate reaction mixture was redetermined to insure that flocculation was not due to iso-electric precipitation of the protein. The addition of sodium chloride did not inhibit precipitation, in distinction to the observed effect on the "quellung" reaction.

Discussion. The mechanism of the "quellung" reaction is not clear. Johnson and Dennison[15] felt that the increase in volume of the pneumococcus resulting from "quellung" was greater than the aggregate volume of the antibody molecules adherent to the capsule. They suggested that capsular hydration took place as a result of antigen-antibody combination. Mudd, *et al.,*[16] on the basis of electron microscopy, felt that the pneumococcal capsule consists of a gel of low density that reacts with homologous immune serum to produce increased thickness and density of capsular gel. Kempf and Nungester[6] noted that a 2 molar solution of sodium chloride did not prevent or reverse the "quellung" reaction to specific serum. The enlarged capsules disappeared on washing several times in water, only to reappear with the addition of physiological saline solution.

Summary. The experiments outlined indicate that pneumococcal polysaccharides combine with various proteins at pH 4.0 to form

15 Johnson, F. H., and Dennison, W. L., J. Immunol., 1944, 48, 317.

16 Mudd, S., Heinmets, F., and Anderson, T. F., J. Exp. Med., 1943, 78, 327.

insoluble aggregates. This reaction is one of loose·combination, since it can be reversed by readjusting the pH to 7.0. Non-specific capsular swelling of pneumococci can be produced also under these conditions, but, in addition, the protein-pneumococcus mixture at pH 4.0 must be relatively salt-free. It is felt that an altered state of the capsular gel is produced by the interaction of protein and carbohydrate. In the absence of ionized salts, the polysaccharide-protein gel becomes increasingly hydrophilic and therefore capable of enlarging to produce the "quellung" reaction. Both specific and the described acid-protein quellung can be produced in the absence óf calcium in distinction to Lofstrom's non-specific "quellung" phenomenon.

16180

Effect of Alloxan Diabetes on Reproduction in the Rat.*

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The administration of alloxan to experimental animals will produce a condition analogous to diabetes mellitus in man.[1,2,3] The principal effect of the drug is a selective necrosis of the islets of Langerhans in the pancreas, although there is slight damage to other tissues.[4,5] The experimental diabetes may be transitory in character and the animal recover, or it may be permanent, becoming progressively more severe until the animal succumbs. The dose of the alloxan administered is the most important factor in the ultimate course of the diabetic state.

Friedgood and Miller[6] injected alloxan 4 days before parturition and obtained normal litters. This observation suggests that terminal diabetes is not sufficient to interfere with the development of fetuses. The high incidence of pregnancy complications in human diabetics prompted us to restudy the problem and the following experiments were designed to provide additional pertinent information.

Methods. The objectives of these experiments were three-fold: (1) to determine the effect of alloxan diabetes on cyclical activity and fertility in the rat, (2) to study reproduction in rats made diabetic early in pregnancy and (3) to evaluate the course of pregnancy and labor in the rat with diabetes controlled adequately by insulin.

Young mature female rats, weighing 175 to 250 g, of the inbred Sprague-Dawley stock raised and maintained in our laboratory were used throughout these studies. They were given free access to a balanced diet which was supplemented by milk as well as wheat germ oil once a week. Sex cycles were followed routinely employing the standard vaginal

1 Bailey, C. C., and Baily, O. T., *J. A. M. A.,* 1943, **122**, 1165.

2 Dunn, J. S., and McLetchie, N. G. B., *Lancet,* 1943, **2**, 384.

3 Goldner, M. G., and Gomori, G., *Endocrinology,* 1943, **33**, 297.

4 Brunschwig, A., and Allen, J. G., *Cancer Res.,* 1944, 4, 45.

5 Dunn, J. S., Sheehan, H. L., and McLetchie, N. G. B., *Lancet,* 1943, 1, 484.

6 Friedgood, C. E., and Miller, A. A., PROC. SOC. EXP. BIOL. AND MED., 1945, 59, 61.

smear technique. The animals were mated by introducing them to males of known fertility and the onset of gestation noted by the presence of spermatozoa in the vaginal smear. It was further checked by the appearance of the placental sign on the twelfth day of the gestation.

Fasting blood sugar levels on venous blood from the tail were determined at 2- or 3-day intervals throughout these experiments by Somogyi's micro-modification of the Schaffer and Hartmann method. The animals were placed in metabolism cages during the day at least 4 hours prior to the blood sugar determinations in order to insure fasting levels. In those animals receiving insulin for the control of the hyperglycemia, qualitative sugar determinations on the urine at frequent intervals made it possible to follow the course of the diabetes.

Cyclical activity was studied in the first group of 6 animals. After a baseline of normal estrous cycles was established for each animal, it was injected with alloxan intravenously. The vaginal smears were followed for a sufficiently long period so that the character of the diabetes induced in the animal could be established and the effect of the hyperglycemia on estrous cycles studied. Varying amounts of alloxan were used. Some of these animals were subsequently mated and their pregnancies followed.

A second group of 49 normal female rats was placed with males and as soon as pregnancy was diagnosed by the appearance of spermatozoa in the vaginal smear they were segregated. Prior to the fifth day of the pregnancy they were injected with varying amounts of alloxan. Blood sugar determinations were made on alternate days throughout their pregnancies. Three of these animals were followed through a second pregnancy. The animals were carefully observed for the accidents of pregnancy.

A third group of 7 rats was made diabetic by alloxan. When it was certain that the diabetes was permanent in character, the hyperglycemia was controlled by insulin. These animals were allowed to mate and the resultant pregnancies carefully studied.

Results. The Effect of Alloxan Diabetes on the Estrous Cycle. After the normal estrous cycle had been established in 6 rats, each received 40 mg of alloxan per kilo of body weight. One animal died 3 days after the injection at which time the fasting blood sugar value was 379 mg %. Two of the animals developed a transient diabetes only and in these the blood sugar levels returned to the normal by the eighth day. The hyperglycemia in these animals reached their highest values, 160 and 207 mg %, on the fifth day following the administration of alloxan. The remaining 3 animals developed permanent alloxan diabetes. Fasting blood sugar levels ranged from 288 to 360 mg % throughout the period of observation.

Following the development of hyperglycemia the estrous pattern changed. Estrous smears occurred with considerable irregularity and the interval was prolonged. The normal 4 or 5 day pattern was replaced by one of 9 to 12 days, the animal remaining in the diestrous condition the greater part of each cycle.

Effect of Alloxan on Mating. Nine diabetic animals in whom blood sugar levels vary from 225 to 350 mg % were placed with males of known fertility. When estrus occurred these animals mated normally as evidenced by the presence of spermatozoa in the vaginal smear. However, the irregularity of the estrous cycles and the prolonged interval between estrous periods lengthened the time necessary for mating.

Three of the animals died 4 or 5 days after spermatozoa were found in the vagina. These animals had normal gestations and their deaths were probably due to the diabetes. Two rats who had hyperglycemia prior to pregnancy developed normal blood sugar levels during the gestation. These 2 animals carried their pregnancies to term and delivered normal litters uneventfully. The remaining 4 animals continued to exhibit a marked hyperglycemia with levels ranging from 210 to 360 mg %. The placental sign appeared on the twelfth day of the gestation. However, at the end of their pregnancies they delivered macerated placentas and no fetuses.

TABLE I.

Summary of Diabetic Rats Treated with Protamine Zinc Insulin. Note the lack of difference in size of litters or weights of fetuses between normal and diabetic animals

Rat No.	Units of Protamine zinc insulin/day	No. of fetuses in litter	Sex		Avg wt, g		Blood sugar P.P. No insulin mg.%	Remarks		
			M	F	M	F				
372	0.75	9	4	5	6.2	4.5	215	F.B.S. 2 days postpartum		
470	0.75	5	2	3	5.0	4.0	373	,,	,,	,,
471	0.75	11	4	7	6.2	5.0	360	,,	,,	,,
580	0.75	4	2	2	5.5	6.0	270	,,	,,	,,
269	0.75	5	2	3	6.5	6.0	399	,,	,,	,,
183	0	9	4	5	6.2	6.2	Normal	Breeding stock	,,	,,
112	0	6	4	2	5.2	5.0	,,	,,	,,	,,
92	0	10	4	6	5.0	5.0	,,	,,	,,	
100	0	5	2	3	6.7	5.6	,,	,,	,,	
186	0	4	2	2	5.6	5.6	,,	,,	,,	

Effect of Alloxan on Pregnancy. A total of 49 pregnant rats were injected intravenously with alloxan, the amount ranging from 30 to 60 mg per kilo, prior to the fifth day of gestation. The most satisfactory dose from the viewpoint of developing a permanent diabetes was 40 mg per kilo. Twenty-seven of these animals died at various stages of their pregnancies and they were studied postmortem. Fourteen of the animals were sacrificed at various periods in their pregnancies in order to provide fresh material for histologic study. Five of the animals survived their pregnancies and delivered macerated placentas at term. The remaining 2 animals did not maintain their hyperglycemia during the pregnancy and they were sacrificed on the ninth and fourteenth days of the gestation. Their pregnancies were progressing uneventfully.

Gross and histologic examinations of the reproductive organs of the diabetic rats revealed typical changes in the entire group. The pregnancies progressed normally until the twelfth day of the gestation. The fetuses and the placentas were normal. Following this period the fetuses died and were slowly absorbed. Necrotic fetuses in various stages of degeneration and absorption were present in the animals sacrificed at varying periods of pregnancy. (Fig. 1.) The placentas continued to grow after fetal death and remained attached to the uterine wall until separated during parturition. The size of the placenta at term was distinctly smaller than the normal placenta probably because of the absence of the fetus. The placentas were delivered at term after an uneventful parturition and all visible remnants of the fetus had disappeared.

These experiments provided proof that the rat made permanently diabetic by the administration of alloxan very early in pregnancy could not deliver a living litter at term because of fetal death prior to the twelfth day. The next step was to see if the control of the hyperglycemia by insulin would result in normal pregnancies. A group of 7 rats was made diabetic by the intravenous injection of 40 mg of alloxan per kilo of body weight. Their fasting blood sugar levels ranged from 253 to 458 mg %. Two weeks later when it was made certain that the diabetes was permanent the animals were placed on protamine zinc insulin, the amount administered being controlled by daily qualitative urine sugar determinations. When the diabetes was controlled by the insulin, the animals were allowed to mate and become pregnant.

The most striking result following the control of the diabetes by insulin was the resumption of normal, regular estrous cycles. The animals became pregnant, carried through to term uneventfully and delivered normal living litters. Two of these animals died at term and subsequent autopsy examinations did not reveal the cause for the deaths. The placentas and the young were normal.

Insulin therapy was discontinued several days after delivery in order to determine the status of the diabetic state. In all instances blood sugar values returned to their previous levels at which they continued as long as

FIG. 1.
Photomicrographs through gravid uteri. A. Permanently diabetic rat at 16 days of gestation. Note the necrotic condition of the fetus. B. Rat with transient diabetes at 15 days gestation. The diabetes lasted 7 days. The fetal structures appear normal. C. Control animal at 14 days gestation.

no insulin was administered. The pregnancies and the insulin therapy did not alter the diabetes. (Table I).

Discussion. Alloxan diabetes in the rat alters the normal cycles, decreases fertility because of this change and affects pregnancy seriously. It is important to make certain that the permanent diabetic state has been induced. Transitory hyperglycemia is a frequent result following an inadequate amount of alloxan. Some of the animals used in these experiments recovered from their diabetic state early in pregnancy and subsequently delivered normal litters at term. Fasting blood sugar levels vary considerably and in the animals used in this study they ranged from 186 to 425 mg %.

Living litters were delivered in none of these diabetic animals. Characteristically, the pregnancy progressed normally until the midpoint of the gestation when the fetus succumbed, following which it was slowly absorbed so that it was absent at term. The placentas remained attached and were delivered at term following a fairly normal parturition.

These observations suggest that terminal diabetes is not sufficient to interfere with the development of the fetuses and that the critical period for the fetus was midpregnancy.

Is it the hyperglycemia that interferes with the estrous cycles and normal gestation in the rat? If the effect of insulin is simply the control of the hyperglycemia, this may be so. A group of diabetic rats were adequately controlled by insulin following which the estrous cycles returned to normal, they conceived and delivered normal living litters at term. The weight of the individual fetus and the gross weight of the litter compared favorably with normal control animals in the colony. It is possible that insulin does more than neutralize excessive blood sugar and diabetes represents more than an altered carbohydrate metabolism.

Summary and Conclusions. The effects of alloxan diabetes on ovarian activity and pregnancy were studied in a series of 63 adult female rats. The development of permanent hyperglycemia resulted in an alteration of the normal estrous pattern, so that the intervals were greatly prolonged. Pregnancy progressed normally until about the twelfth day following which the fetus died and was slowly absorbed. The placentas were retained and were delivered on the day of parturition. The adequate treatment of the alloxan diabetes by insulin resulted in normal pregnancies and live litters delivered at term.

<div align="center">16181</div>

Effect of Dietary Variations upon the Toxicity of DDT to Rats or Mice.*

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The effectiveness of toxic agents in animals frequently depends upon the composition of the basal diet fed. Thus a high protein diet has been found to protect the liver against such diverse toxic agents as arsphenamine,[1] p - dimethylaminoazobenzene,[2] chloroform,[3] and selenized wheat.[4] Methionine is effective against several of these agents.[5] Another nutrient reported to modify toxicity is fat. When rats were fed trinitrotoluene (TNT) in diets high in protein or carbohydrates, little or no ill effects were observed, although definite toxic effects resulted when a high fat diet was fed.[6] On the other hand, mice fed dinitrotoluene (DNT) grew better and lived longer on a high fat diet than when the basal diet was nearly devoid of fat.[7] Vitamin C is reported to be of value in the prevention and treatment of toxic effects of TNT in munition workers[8] and to aid in the detoxification of aniline in rabbits and mice.[9] Chloronitrobenzene toxicity has been found to be aug-

mented by fats and oils, as well as by alcohol,[10] and alcohol also increases the toxicity of dinitrotoluene.[7,10] These observations and the widespread use of DDT (2,2 bis(p-chlorophenyl) 1,1,1 trichloroethane) as an insecticide raised the question whether the toxicity of this compound in higher animals might not also vary with the composition of the basal diet fed.

Experimental. Methods. Most of the experiments were performed on young mice, but adult mice and both young and adult rats were also used in certain studies. The animals were kept in screened cages in groups of 3 to 6 and they were fed the synthetic or semi-synthetic diets listed in Table I, or similar diets in which the percentage of fat or protein was altered isocalorically at the expense of the carbohydrate. The animals were weighed periodically and the toxicity symptoms noted. The DDT used in these experiments was prepared by the method of Darling[11] and recrystallized twice from ethyl alcohol.

Results. In preliminary studies, DDT was incorporated into the yeast diet I and fed to young mice in graded concentrations ranging from 0.01% to 0.3%. At concentrations of 0.05% and over, the mice rapidly developed toxicity symptoms and died within 2 weeks or less. At the lowest concentration, 0.01% they survived for a 9-week period. At the intermediate concentrations, 0.03% and 0.04%, approximately one-half of the animals lived for 4 weeks, depending upon the concentration of the insecticide and the size of the mice. Older mice resisted the effects of the toxic agent somewhat better than younger ones. The symptoms of toxicity were similar

1 Messinger, W. J., and Hawkins, W. B., *Am. J. Med. Sci.*, 1940, **199**, 216.

2 Gyorgy, P., Poling, E. C., and Goldblatt, H., Proc. Soc. Exp. Biol. and Med., 1941, **47**, 41.

3 Miller, L. L., and Wipple, G. H., *Am. J. Med. Sci.*, 1940, **199**, 204.

4 Moxon, A. L., *S. Dak. Exp. Sta. Bull.* No. 311, p. 50, 1937.

5 Miller, L. L., and Wipple, G. H., *J. Exp. Med.*, 1942, **76**, 421.

6 Himsworth, H. P., and Glynn, L. E., *Clin. Sci. and Heart*, 1942, **4**, 421.

7 Clayton, C. C., and Baumann, C. A., *Arch. Biochem.*, 1944, **5**, 115.

8 Holmes, H. N., *Science*, 1942, **96**, 384.

9 Rubanovskaya, A. A., *Farmakol. i Taksikol.*, 1945, **8**, No. 4, 43, *Chem. Abst.*, 1946, **40**, 6686.

10 Von Oettingen, W. F., *Pub. Health Bull.* 271, U. S. Public Health Service, 1941.

11 Darling, S. F., *J. Chem. Education*, 1945, **22**, 170.

TABLE I.
Composition of the Diets Fed.

Yeast diet (I)		Synthetic diet (II)	
	g		g
Crude casein	18	Crude casein	20
Wesson's salts[14]	4	Wesson's salts	4
Brewers yeast	8	Celluflour	2
Corn oil	5	Corn oil	5
Dextrin	70	Dextrin	69
			mg per 100 g of diet
		Pyridoxine hydrochloride	0.5
		Thiamine chloride	0.6
		Calcium pantothenate	2.0
		Riboflavin	0.6
		Nicotinic acid	1.0
		Choline chloride	100.0
		Inositol	50.0
		p-Aminobenzoic acid	30.0
		Cystine	100.0

to those described by others[12,13] and were characterized by hyperexcitability, followed by a jerking or retraction of the head, which developed into a general tremor that increased in intensity until there was complete loss of control, convulsions, and death. Alopecia was common in certain series. The compound did not exert any appreciable effect on the rate of growth until the tremors became sufficiently severe to interfere with the ability of the mice to eat. In general, rats responded to DDT like mice, although higher concentrations of DDT were necessary before deaths resulted in a comparable period of time: 0.15% of DDT was about as effective in a rat as 0.04% in the mouse.

The effect of DDT against either species depended upon the percentage of fat in the basal diet (Table II). Mice invariably developed toxic symptoms and died sooner when fed a diet containing 5% or more of fat than when fed a diet containing only 0.5% of fat; this was observed in both young and older mice and in series in which the concentration of DDT was 0.03% or 0.04% (Table II). Similar results were obtained with rats fed 0.15% of DDT (Table II). In other experiments, series of different fats

were fed to mice at a 15% level in diets containing 0.03% or 0.04% of DDT. All fats tested appeared to increase the sensitivity of mice to DDT at this level. Mice fed a completely saturated fat, hydrogenated coconut oil, developed tremors and died at about the same time as those ingesting the moderately saturated fats, butter or lard, or the highly unsaturated fats, peanut oil or corn oil. The effect of the fat was not modified by the addition of 0.5% of cholesterol.

A reduction in the level of protein in the diet to 10% apparently decreased the resistance of the mice to DDT. This effect was noted whether the percentage of fat in the diet was high or low (Table II). When the level of protein in the diet was increased above 20%, the results were variable although some protective action against DDT appeared in certain series.

Discussion. The greater sensitivity of animals to DDT fed in a high fat diet as compared to one low in fat seems to be primarily due to a greater efficiency of absorption of DDT on the high fat diets. DDT is readily soluble in fat and tends to concentrate in the fat depots of animals exposed to this insecticide, e.g., in rats,[15] cows,[16] and dogs.[17]

12 Woodard, G., Nelson, A. A., and Calvery, H. O., J. Pharm. Exp. Ther., 1944, 82, 152.

13 Smith, M. I., and Stohlman, E. F., Public Health Rep., 1944, 59, 984.

14 Wesson, L. G., Science, 1932, 75, 339.

15 Woodard, G., and Ofner, R. R., Fed. Proc., 1946, 5, 215.

16 Wilson, H. F., Allen, N. N., Bohstedt, G., Betheil, J., and Lardy, H. A., J. Econ. Entomol., 1946, 39, 801.

TABLE II.

Effect of the Level of Fat and Protein in the Diet upon the Survival of Mice and Rats Fed DDT. Diet II.

Casein, %	Corn oil, %	Number of animals alive at:						
		0 wk	1 wk	2 wks	3 wks	4 wks	5 wks	6 wks
		0.03% DDT, young mice.						
20	0.5	4	4	4	4	4	4	4
20	5	4	4	4	4	4	3	2
20	15	4	4	3	2	2	1	0
		0.03% DDT, young mice.						
20	0.5	5	5	5	5	3	3	
20	15	5	5	5	4	2	1	
10	15	5	5	4	1	1	0	
50	15	5	5	4	4	4	4	
		0.04% DDT, young mice.						
20	0.5	10	10	7	6			
20	5	4	4	1	1			
20	15	10	7	3	0			
10	0.5	6	5	1	0			
10	15	6	6	1	0			
		0.04% DDT, adult mice.						
20	0.5	4	4	4	4	4	4	4
20	5	4	3	3	3	3	2	2
20	15	4	4	4	4	4	3	2
20	25	4	3	3	2	1	1	1
10	15	4	3	0	—	—	—	—
50	15	4	3	3	3	3	2	2
		0.15% DDT, young rats.						
20	0.5	6	6	5	5	5		
20	5	6	2	0	—	—		
20	15	6	4	1	0	—		

More DDT accumulated in the fat of dogs when the compound was fed in corn oil solution than as a solid[17] and the symptoms of toxicity due to injected DDT were also more severe,[12,13] when an oil solution was used. Factors affecting the translocation of DDT within the body are probably more important in determining resistance to DDT than to other poisons. DDT does not appear to be rapidly detoxified in the body[18] and its association with fat may be an important mechanism for its removal from the general circulation. However, at least one metabolic derivative of DDT (di(p-chlorophenyl) acetic acid) has been identified in the urine of rabbits,[19] and fat may affect the speed with which such metabolites are formed. Dietary fat seems to influence the toxicity of certain nitro compounds.[6,7]

The mouse appears to be more sensitive to DDT than the rat, for on comparable diets tremors produced in mice fed 0.03% to 0.04% of DDT were similar to those in rats fed 0.10% to 0.15% of DDT. In other words the rat can tolerate 3 times the concentration tolerated by the mouse. However, the mouse consumes roughly 2 to 3 times as much food per unit of body weight as the rat and, hence, the amounts of ingested DDT per gram of body substance required for the production of tremors were quite similar in the two species. Indeed, it has been reported that the rat is somewhat more susceptible than the mouse to injected DDT or to large single oral doses.[12] Nevertheless, the present data indicate that the mouse has definite advantages as an animal for the bio-assay of foodstuffs exposed to the insecticide.

Summary. Mice fed 0.03% to 0.04% of DDT in a medium fat diet (5%) developed toxic symptoms and died; 0.10% to 0.15% of DDT in the same diet was necessary to

17 Woodard, G., Ofner, R. R., and Montgomery, C. M., *Science*, 1945, **102**, 177.

18 Philips, F. S., and Gilman, A., *J. Pharm. and Exp. Therap.*, 1946, **86**, 213.

19 White, W. C., and Sweeney, T. R., *Public Health Rep.*, 1945, **60**, 66.

produce a similar toxicity in the rat.

A reduction in the level of dietary fat to 0.5% decreased the toxicity of DDT in both species; when the diet contained 15% of fat, the symptoms of toxicity were aggravated. Several different types of fat were essentially equal in aggravating the toxicity.

On a low protein diet (10%) the toxicity of DDT was increased somewhat; whereas on a high protein diet (30%) the effects were variable.

16182 P

Endocrine Interrelationship and Spontaneous Tumors of the Adrenal Cortex in NH Mice.*

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Adenomas of the adrenal cortex appear in several inbred stocks of mice following gonadectomy.[1,2,3] In certain stocks, however, the phenomenon does not occur following removal of the primary sex glands.[3,4] In only one strain have similar adenomas been observed to appear spontaneously in non-castrate animals.[5] This is the NH stock† (Minnesota subline); tumors occur primarily in females. In these mice adenomas of the same type which develop spontaneously can be induced to appear precociously by gonadectomy.[6] The purposes of the current studies on this stock are to determine first, whether "physiologic castration" is responsible for the induction of spontaneous adenomas of NH females (spontaneous cortical tumors are very rare in males) and, second, to investigate the effect of gonadotrophic hormone on the secretion and development of these tumors.

The vaginal smear which is exhibited by mice with cortical adenomas is typical. The vaginal secretion is copious, relatively watery, and has a high cellular content. The cells are of two general classes—epithelial cells and leukocytes—these are approximately equally distributed. The epithelial cells may be classified as cornified and non-cornified. The ratio of cornified to non-cornified cells in the untreated tumor-bearing animal is 25 to 75.

The estrous cycle was studied in 6 inbred stocks of mice (CBA, dba, Balb, Strong A, F., NH). In general, it was found that the estrous cycle lengthened as the animals became infertile. This was observed to occur earlier in the NH stock than in any of the others except the F mice. Females of all stocks except the NH and F were cycling beyond one year of age. The F females (10 mice) exhibited a castrate smear by one year of age but did not develop cortical adenomas. If castrated early in life these mice showed only a minimum tendency towards adenoma development, suggesting that the end organ is not sensitive to the stimulus towards tumor formation. (CBA, dba, and Balb mice exhibit cortical adenomas within 3-5 months after gonadectomy). In most cases the NH females (43 mice) did not

1 Woolley, G., Fekete, E., and Little, C. C., *Endocrinology*, 1941, 28, 341.

2 Gardner, W. U., *Cancer Research*, 1941, 1, 632.

3 Smith, F. W., *Science*, 1945, 101, 279.

4 Woolley, G., Fekete, E., and Little, C. C., PROC. SOC. EXP. BIOL. AND MED., 1940, 45, 796.

5 Kirschbaum, A., Frantz, M. F., and Williams, W. L., *Cancer Research*, 1946, 6, 707.

6 Kirschbaum, A., and Frantz, M. F., unpublished data.

develop castrate smears, but developed the smear characteristic of mice bearing cortical tumors within a few cycles after lengthening of the cycle (within 30-50 days after the last litter the vaginal smear picture typical of cortical adenoma appeared).

Histologic study of the ovary revealed that at one year of age Graafian follicles were present in the ovaries of all stocks except the NH. (The reason for lack of cycles in the F strain is not clear). The evidence suggests that physiologic castration may be a factor in the development of adenomas in the NH females. It is of interest that the smear typical of tumor formation appeared before there was histologic evidence for a well developed tumor. Sections of the vagina showed extreme cornification. It is probable that the histologically unaltered NH adrenal may secrete estrogen in quantity sufficient to stimulate the female reproductive tract.

When only the ovaries were removed from 6 tumor-bearing mice, the vaginal smear picture was unaltered. When the adrenals alone were removed‡ (ovaries remaining) then the vaginal smear became of castrate type within 5 days (7 cases). This constitutes proof that the adrenal tumors were the primary source of estrogen.

The effect of gonadotrophic hormone was studied on the same mice (adrenalectomized or ovariectomized). One hundred and ten international units (5 units daily for 22 successive days) of pregnant mare serum§ elicited no change in the castrate smear of the adrenalectomized NH females, indicating the refractoriness of the ovaries to gonadotrophic hormone (the ovaries of older mice of other stocks were readily stimulated). The same amount of this hormone, when given to 3 ovariectomized mice (with adrenal tumors) induced a change in the vaginal smear picture: (1) the quantity of secretion was increased, and (2) the ratio of cornified to noncornified cells was altered from 25:75 to at least 50:50. The number of leukocytes remained the same. It would appear that gonadotrophic hormone can influence sex-hormone secretion of an adenoma of the adrenal cortex.

Summary. The occurrence of spontaneous estrogen-secreting tumors of the adrenal cortex can be correlated with early cessation of ovarian activity in the NH stock. Estrogen is probably secreted by the adrenal cortex even preceding adenoma formation. Gonadotrophic hormone enhanced estrogenic secretory activity of cortical adenomas.

‡ Animals maintained on aqueous cortical extract—Upjohn.

§ Gonadogen—Upjohn.

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Early Effect of X-rays on Ovaries of Normal and Adrenalectomized Rats.*

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In a previous paper[1] we described the changes occurring in the rat ovary during the first few hours after irradiation with X-rays.

These alterations are characterized by the presence of numerous pycnotic nuclei of the follicular cells of the granular layer of the follicle.

We are able to distinguish between pycnotic nuclei induced by irradiation and those normally found in certain ovarian follicles, by showing that pycnotic nuclei of granular cells

1 Halberstaedter, L., and Ickowicz, M., *Radiology*, 1947, **48**, 369.
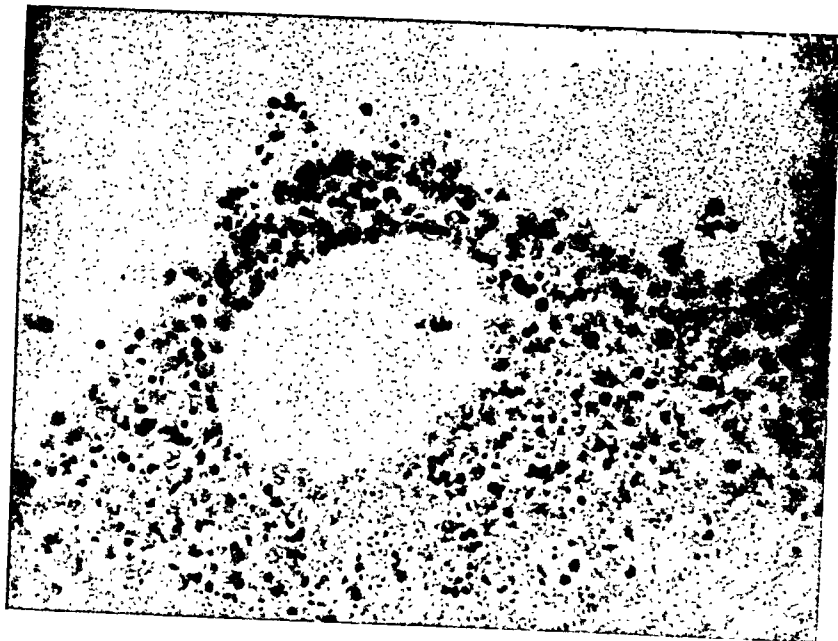
Fig. 1.
Adrenalectomized rat. Graafian follicle with numerous pycnotic nuclei.
The nucleus of the ovum is in mitosis. × 640.

in the ovaries of non-irradiated rats occur only in follicles in an advanced stage of development, and whose ovules are already dividing, whereas after X-irradiation numerous pycnotic nuclei are also found in very young follicles and in those whose ovules are in the resting state. In studying the earliest ovarian lesions induced by irradiation it is therefore necessary to examine the entire organ microscopically by means of complete serial sectioning, in order to determine the condition of the ovule.

In addition, we have observed that during the first few hours after irradiation ovarian lesions are limited particularly to follicular cells, while no lesions are to be seen in the other elements of the ovary.

The object of the present study was to determine the effect of adrenalectomy on pycnosis of the follicular cells of the rat ovary during the first few hours after X-ray irradiation.

Methods and Technique. Our experiments on the ovaries of highly inbred albino rats (weighing approximately 150 g) fall into the following 3 categories:

A. Adrenalectomized rats. These animals were killed 24 hours after double adrenalectomy.

B. Irradiated rats. These animals were irradiated over the abdominal region with 2000 r and were killed 4 hours after irradiation.

C. Adrenalectomized and irradiated rats. These animals were adrenalectomized and were irradiated over the abdominal region with 2000 r 24 hours later. They were killed 4 hours after irradiation.

Irradiation was delivered by a Machlett X-ray tube operated on a multivolt apparatus of 150 K.V., Al. 0.5 mm, distance 30 cm, intensity 100 r/minute. The ovaries were fixed in Bouin's fluid. Complete serial sections were prepared and stained with iron hematoxylin and eosin, according to the method of Masson.

Histological Description. The following microscopic description is based on composite data for each group of rats, with special emphasis on pycnotic nuclei of the follicular cells and their relationship to the state of the ovule.

A. *Adrenalectomized rats.* Follicles at differ-

FIG. 2.
Rat irradiated with 2,000 r. Young follicle with pycnotic nuclei. × 450.
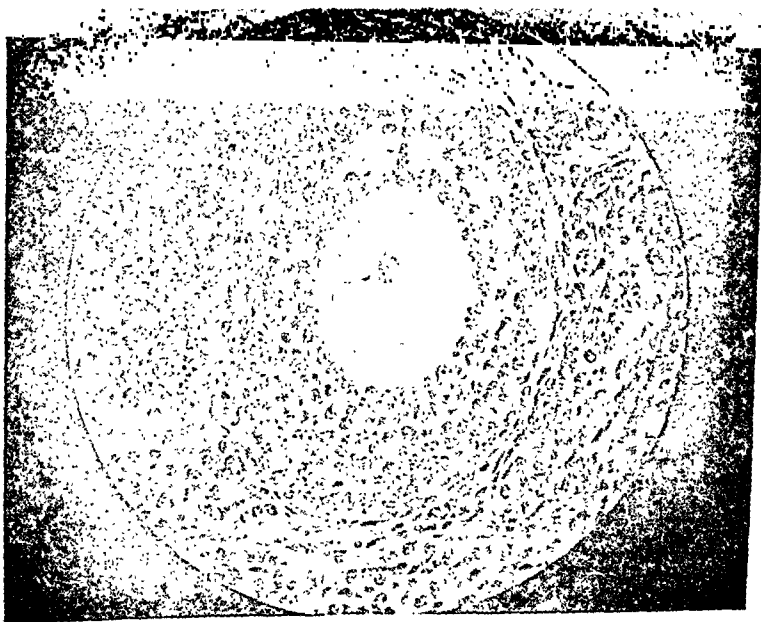
FIG. 3.
Rat adrenalectomized and irradiated with 2,000 r. Young follicle without pycnotic nuclei. × 450.

ent developmental stages were found: Graafian follicles, as well as stratified and young ones. The Graafian follicles of varying stages may be divided into 2 groups: a) Those with pycnotic nuclei in the granular layer, and with a dividing ovule (Fig. 1); and b) those without pycnotic nuclei or with very few in the granular layer and with a resting ovule.

We have, however, come across one ovary with Graafian follicles containing resting ovules, but with a considerable number of pycnotic nuclei in the granular layer. Such follicles are very unusual. The presence of pycnotic nuclei in the granular layer of young follicles (from primary to stratified follicles) is likewise exceptional. On the contrary, mitoses are frequently seen in the granular layer of young follicles.

Furthermore, no changes associated with adrenalectomy have been observed in the other ovarian elements, such as the atresic follicles, corpora lutea, blood vessels or the general stroma. Thus, the ovaries of adrenalectomized rats 24 hours after the operation do not differ significantly from normal rat ovaries.

B. *Irradiated rats.* Microscopic examination 4 hours after X-ray irradiation revealed extensive pycnotic lesions of the follicular cells in all advanced as well as in young follicles (Fig. 2). The number of atresic follicles seems to have increased. However, very rarely, some Graafian follicles appear to be resistant to X-rays: but these are exceptional cases and do not alter the general picture of the massive pycnotic lesion observed in the ovaries 4 hours after irradiation with an adequate dose.

C. *Adrenalectomized and irradiated rats.* Microscopically no pycnotic nuclei were seen in cells of the granular layer in young follicles (Fig. 3) and in the majority of Graafian follicles containing resting nuclei. As in the ovaries of normal rats, numerous pycnotic nuclei were seen in follicles with dividing ovules. However, we have also observed pycnotic nuclei in certain follicles containing resting ovules in the ovaries of one rat. But these lesions were, nevertheless, infinitely less

pronounced than those observed in irradiated rats without previous adrenalectomy. No changes were seen in the other ovarian elements.

Discussion. Our experiments demonstrate that adrenalectomy inhibits the appearance of pycnotic lesions in the rat ovary 4 hours after X-ray irradiation. These observations are analogous to those obtained with lymphatic tissues and described by Leblond and Segal[2] and by Halberstaedter and Ickowicz.[3]

The small cells in the granular layer of the ovary appear to react as do lymphocytes. We are as yet unable to tell just how far this analogy may be drawn, since our experiments deal only with pycnotic lesions due to X-ray irradiation. Actually, observations on lymphocytes have shown that these cells are extremely sensitive not only to X-rays but also to a number of harmful agents, both chemical and physical[4] and even to so simple a trauma of the animal organism as an incision of the skin.[5] In any case our experiments clearly demonstrate that under certain conditions, such as X-ray irradiation for example, the adrenal glands influence the pycnotic reaction of the cells of the granular layer of the ovary.

An important point brought to light by our experiments is the fact that adrenalectomy inhibits pycnosis of the granular cells of the ovary even after direct irradiation of the organ. This constitutes a special phenomenon, since in the case of lymphocytes adrenalectomy seems to influence the ensuing pycnosis caused only by the indirect effect of the irradiation.[2,3] So far we have not yet elucidated the question of the influence of adrenalectomy on the indirect effect of irradiation on the ovary.

Summary. The pycnotic lesions of the cells in the granular layer of the ovarian follicles of rats 4 hours after X-ray irradiation are inhibited by previous adrenalectomy.

[2] Leblond, C. P., and Segal, G., *J. Roentgenol. and Rad.*, 1942, 47, 302.

[3] Halberstaedter, L., and Ickowicz, M., *Radiologia Clinica*, 1947, 16, 240.

[4] Selye, H., *J. Clin. Endocrin.*, 1946, 6, 117.

[5] Ickowicz, M., *Radiologia Clinica*, 1947, 16, 231.

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Aluminum Penicillin in Mouse Protection Tests.

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In searching for a preparation of slowly absorbed penicillin that would give satisfactory results, the amorphous aluminum salt of penicillin was prepared in our laboratories. As expected, this penicillin was found to be highly insoluble in water. It was therefore thought that if it could be suspended in a suitable non-aqueous medium a single daily parenteral injection would give prolonged therapeutic activity. Peanut oil was selected as the vehicle for suspension.

It is the purpose of this report to show the protective effect of aluminum penicillin in peanut oil on mice infected with *Diplococcus pneumoniae*. Clinical reports to be published elsewhere will describe blood level determinations and therapeutic efficacy of aluminum penicillin in human subjects. However, when 300,000 units of aluminum penicillin in peanut oil is given intramuscularly to human subjects, blood levels of 0.03 units per ml of serum were found to persist from 12 to 24 hours. The concentration of penicillin in body fluid of treated mice has not been determined.

Procedure. 18 to 22 g mice were infected intraperitoneally with 0.5 ml of a 10^{-5} dilution of an 18-hour broth culture of *Diplococcus pneumoniae* Type I (SVi strain) containing 1000 MLD. Two hours later treatment was begun as indicated in Table I by injecting 0.1 ml of the suspension of penicillin in peanut oil intramuscularly. The suspensions were made by diluting penicillin in oil and mixing in a Waring blendor to the proper unitage. All dilutions were assayed before use. Treatment was repeated daily for 4 days and mice were held until the eighth day when the results were finally determined.

Under the conditions of these experiments, the effectiveness of aluminum penicillin was compared with calcium penicillin in peanut oil and sodium crystalline penicillin in peanut oil and beeswax (4.8 W/V). Untreated, infected controls died within 36 hours. The results are shown in Table I.

From the above data it is determined that the PD_{50} for each of the compounds tested is:

(1) Aluminum penicillin in peanut oil   34 units
(2) Sodium penicillin in oil and wax   40 units
(3) Calcium penicillin in oil   95 units

This suggests that under the conditions described, aluminum penicillin is superior in protective ability to either of the other compounds tested. This may be considered surprising in view of the reference to human blood levels in which aluminum penicillin does not appear to remain in the blood stream as long as peanut oil and beeswax preparations. Further studies are in progress to explain this seeming inconsistency, however, it seems appropriate at this time to propose an explanation. Aluminum penicillin being very insoluble in water must, for purposes of assay or utilization by the animal body, be converted into a soluble salt, *e.g.* sodium penicillin. This process takes place *in vivo* slowly and at a rate proportional to the amount of aluminum penicillin present in tissues. For this reason blood serum levels run a curve having a peak about one hour after injection, slowly diminishing in several hours to a level below an amount that can be measured by present methods for determining its concentration. This lower concentration, though at present not considered therapeutic, may be adequate to hold the invading organisms in check until the body defenses can overcome the infection.

To further study the prolonged action of aluminum penicillin in oil against pneumococcal infections in mice, infected animals were treated with a single intramuscular injection. Results of these protection tests are given in Table II.

Conclusion. Aluminum penicillin in oil ef-

TABLE I.
Protection with Multiple Doses of Penicillin.

Preparation	Units of penicillin		Survivors		
	Daily	Total 4 days	No. of mice	8 days	% protection
Aluminum penicillin in oil	200	800	80	80	100
	150	600	50	50	100
	100	400	90	89	99.0
	75	300	90	88	98.0
	50	200	90	73	81.0
	25	100	90	30	33.2
Sodium crystalline penicillin in oil	200	800	50	48	96.0
and beeswax (4.8 W/V)	100	400	50	48	96.0
	75	300	50	46	92.0
	50	200	50	34	68.0
	25	100	50	11	22.0
Calcium penicillin in oil	200	800	50	37	74.0
	150	600	50	32	64.0
	100	400	50	26	52.0
	75	300	50	21	42.0
	50	200	50	2	4.0
	25	100	30	0	0.0
Controls	No treatment		60	0	0.0

TABLE II.
Protection with a Single Dose of Aluminum Penicillin in Oil.

Preparation	Units of penicillin	No. of mice	Survivors 8 days	% protection
Aluminum penicillin in oil	400	50	50	100
	200	50	50	100
	150	50	46	92
	100	50	47	94
	75	50	45	90
Control	None	30	All dead 36 hrs	

fectively combats induced pneumococcus infection in mice. Compared with calcium penicillin in oil or sodium penicillin in peanut oil and beeswax preparation, the aluminum penicillin in oil is more effective on a unit for unit basis.

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